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Genetic background influences the effect of neurokinin-1 receptor "knockout" in the mouse

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Abstract

Strains of mice used in laboratory research differ markedly at a molecular, anatomical and behavioural level. Genetic manipulation in mice has become a widely used tool for the selective study of single genes and has also produced a great diversity of phenotypes. However, the interaction between manipulated genes and background strain has been less well examined. Here, the effect of neurokinin-1 (NK1) receptor disruption is shown to depend heavily on the genetic background of the mouse that it is studied in. $NK1^{-/-}$ mice have previously been shown to display an anxiolytic and antidepressant-like phenotype, as well as having reduced sensitivity to the rewarding properties of the opiates. Here, after being transferred onto a C57BL/6 (B6) background, the anxiolytic effect of the mutation is lost, as is the differential response to morphine's locomotor stimulating properties. When the mutation is transferred onto a mixed C57BL/6 x 129/sv (B6:129) background the $NK1^{-/-}$ mouse becomes more sensitive to morphine than wildtype counterparts, although no difference in anxiolysis is evident between the genotypes. Examination of the HPA axis in these animals revealed that B6:129 animals had higher levels of stress-induced corticosterone release than B6. Furthermore, $NK1^{-/-}$ animals on the B6:129 background expressed higher levels of hippocampal glucocorticoid receptor and exhibited more neurogenesis in the hippocampus than wildtype. No differences in these parameters were observed for the genotypes in the B6 strain. Comparison of CRF and cFos mRNAs did not reveal any differences between genotypes in either strain. These results show that there are important epistatic interactions between genetic background and the NK1 receptor. These findings may be relevant for the treatment of anxiety disorders and opiate addiction in the genetically heterogeneous patient population.

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11β-HSD1	11β-Hydroxysteroid dehydrogenase type 1
11-DHC	11-dehydroxycorticosterone
5-HT	5-Hydroxytryptamine; serotonin
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AVP	Arginine vasopressin
B6	C57BL/6
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral nucelus of the amygdala
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
BZ	Benzodiazepine
cAMP	Cyclic adenosine monophosphate
CBG	Capillary blood glucose
CeA	Central nucelus of the amygdala
CNS	Central nervous system
CO_2	Carbon dioxide

COMT	Catecholamine methyl transferase
CPP	Conditioned place preference
CPu	Caudate putamen
CRF	Corticotrophin releasing factor
CSF	Cerebral spinal fluid
D2	DBA/2J
DA	Dopamine
DAB	3,3′-diaminobenzidine tetrahydrochloride
DAG	Diacyl glycerol
DAOA	D-amino acid oxidase activator
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
DRN	Dorsal raphe nucleus
DTT	Dithiothreitol
ECT	Electroconvuslive shock therapy
EDTA	Ethylenediaminetetraacetic acid
EPM	Elevated plus maze
ES cells	Embryonic stem cells
GABA	γ aminobutyric acid
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
HRP	Horseradish peroxidase
i.c.v.	intracerebroventricularly

i.p.	Intraperitioneal
IHC	Immunohistochemistry
IP_3	Inositol-3-phosphate
IRES	Internal ribosome entry site
LC	Locus coeruleus
LDEB	Light/dark exploration box
MANOVA	Multivariate analysis of variance
MAO	Monoamineoxidase inhibitor
MeA	Medial nucleus of the amygdala
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
NA	Noradrenaline
NAcc	Nucleus accumbens
NK1	Neurokinin-1
NMDA	*N*-methyl-D-aspartic acid
NRI	Noradrenaline reuptake inhibitor
NSB	Non-specific binding
OCD	Obsessive compulsive disorder
PAG	Periaquaductal gray
PB	Phosphate buffer
PbN	Parabrachial nucleus
PBS	Phosphate buffered saline
PC2	prohormone convertase
PCR	Polymerase chain reaction

PFA	Paraformaldehyde
PKC	Protein kinase C
PLA_2	phospholipase A_2
PLCβ	Phospholipase Cβ
PNS	Peripheral nervous system
PPT-A	preprotachykinin A
PTSD	Posttraumatic stress disorder
PVDF	Polyvinylidine difluoride
PVN	Paraventricular nucleus of the hypothalamus
QTL	Quantitative trait locus
RGS2	Regulator of G-protein signaling 2
RT	Room temperature
rTdT	Terminal deoxytransferase
RVM	Rostroventral medulla
SA	Self administration
SAP	Stretch attend posture
SEM	Standard error of the mean
SP	Substance P
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TSA	Tyramide signal amplification
VTA	Ventral tegmental area

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Chapter 1

Introduction

The advent of genome engineering in the mouse has led to an explosion in biomedical research in rodents. These studies have been able to investigate specific molecules not just at a cellular or biochemical level but also their role in more complex behaviours. Research using the neurokinin-1 (NK1) receptor knockout mouse demonstrates this trend. The last decade has seen the NK1 receptor implicated in a number of conditions related to pain, motivation and affect, in part via work conducted in mice lacking a functional NK1 receptor. However, during this period NK1 antagonists introduced into clinical trials have failed to live up to their predicted potential as novel antidepressants. The validity of many studies using knockout mice has been questioned because of the failure of different laboratories to reproduce each other's results. Concurrently, our knowledge of molecular genetics has been expanding and the call for standardisation of practice between the laboratories that are using these sophisticated technologies has been increasing.

The majority of experimental work in this thesis is concerned with the effect of transferring the mutation in the NK1 receptor onto a different genetic background and detailing the effects this has on the mouse phenotype. This is an attempt to use these differences between genetic background to understand the role of the NK1 receptor in the behaviours examined.

This chapter briefly reviews the history of laboratory rodents and the approaches that have led to genome engineering in the mouse as well as the associated problems. This is followed by a history of research into substance P and the NK1 receptor

including their known distribution and biological functions with particular reference to nociceptive, affective and addictive behaviours.

1.1 Laboratory research using mice and rats

1.1.1 History

Rodents, particularly mice and rats, have been used in scientific laboratories for many years and have contributed enormously to our understanding of biological processes. Many different strains, both outbred and inbred, have been tested in a plethora of experimental paradigms and are very well characterised (Crawley et al., 1997). Recent advances in molecular genetics have made the use of transgenic and knockout animals commonplace in biomedical science. This has, however, introduced uncertainty into which particular strain of mouse is best suited for the intended work. Coupled with technical considerations involved in the creation of the mice this means that much research using these techniques has been irreproducible or in some cases has even led to contradictory results. In the next section, after an introduction to the differences between mouse strains commonly used, the techniques utilised to create a genetically modified mouse will be described followed by some of the common problems with this process.

1.1.2 Mouse strains

Laboratory mice have had a prominent role in biomedical research for over 100 years and their study has given rise to many insights concerning human disease states. Their mammalian lineage gives them far more relevance to our biology than other organisms such as zebrafish, *Drosophila* or yeast. Breeding is quick and easy to conduct and recently, with advances in genome engineering and the full sequencing of the mouse genome, powerful tools allowing the manipulation of this code have become available (Jockusch et al., 2004). Even preceding their use in science a great

deal of genetic variation existed between mice from around the world. Some of this variation was naturally occurring, arising from migratory patterns, whilst some of it was introduced by human mouse 'fanciers' who both in the Far East and Victorian England kept and traded mice as pets (Wade and Daly, 2005). However, with the advent of laboratory facilities dedicated to breeding mice and the emergence of the field of genetics, different populations or sub-species of the mouse, *Mus musculus*, arose and have become properly characterised.

1.1.3 Inbred and outbred stocks

Laboratory mice can generally be divided into inbred or outbred stocks. The classically inbred mice were developed from small founder populations and include widely used strains such as C57BL/6 (B6), 129 lines and DBA/2J. These mice have lost the polymorphisms in their genome through inbreeding and are homozygous at the vast majority of alleles (Beck et al., 2000). These differ from strains referred to as outbred which retain polymorphisms in their genome (Chia et al., 2005). There are advantages and disadvantages to using inbred or outbred stocks that are important to consider when designing experiments.

1.1.3.1 Outbred mice

The official definition of an outbred mouse stock is one that is " a closed population (for at least four generations) of genetically variable animals that is bred to maintain maximum heterozygosity. " (Festing, 1993). In order to maintain genetic variability it is essential that the colony is maintained at a relatively large size, at least 25 breeding pairs. In small colonies random genetic drift can occur as homozygosities occurring as a result of inbreeding become fixed in a population. This can become particularly problematic if two populations for comparison are maintained separately. This is of particular relevance to work using transgenic animals where, for example, the mutant and wildtype animals may be interbred separately. After several generations

of breeding, the limited gene pool can lead to the genetic background of the two populations diverging as alleles become randomly segregated. This segregation can lead to phenotypic variation between the populations which is independent of the locus being studied (Chia et al., 2005). Although this is also a problem with inbred stocks, the potential for genetic drift is far less.

Outbred stocks are widely used though and do show advantages for some experiments. Similarly to the human population, outbred stocks accumulate many breakpoints in their genome over time, fragmenting the chromosome into mosaic-like portions (Chia et al., 2005). These can be used with quantitative trait loci mapping to identify regions of the chromosome which statistically link to a phenotype of interest (Flint et al., 2005). As an example, breeding of the outbred MF1 mouse to inbred stock has been used to identify regulator of G-protein signaling 2 (RGS2) as a gene influencing anxiety (Yalcin et al., 2004).

Outbred mice are often used to develop mouse models of human conditions due to the high variability between strains and individuals. These features can be used to selectively breed for a phenotype of interest. Among mice generated in this way are those for high activity (Bronikowski et al., 2002), obesity (Horvat et al., 2000), prolificacy and weight (Kirkpatrick et al., 1998) and with different preferences to alcohol (Grahame et al., 1999). Once developed these mice are used to identify the physiological or genetic markers that underlie these conditions.

Another reason why outbred strains may be used is that the genetic variability also contributes to a better breeding performance than many inbred strains. This makes them attractive animals for the development and maintenance of transgenic animals. However, due to the reasons such as genetic drift mentioned above, this should be avoided if possible (Chia et al., 2005).

1.1.3.2 *Inbred mice*

Inbred mice are more commonly used in laboratories than outbred mice. The lower genetic variability, in theory at least, gives rise to less experimental variability whether the trait under examination is behavioural, biochemical or molecular. This reduced variability will generally decrease the sample size required to see an effect in an experiment and so increase the experimental power (Chia et al., 2005). However, the likelihood of seeing an effect also has the potential to be masked or obscured depending on the inbred stock used and its particular modifying genes. As such, the inbred strain may be in some way resistant to a chemical being tested or compensations may render a particular gene redundant, a phenomenon known as epistasis (Crawley et al., 1997). Other problems associated with inbred strains are that the homozygosities that have been selected for may often be deleterious to the animal. As an example B6 mice are known to develop poor auditory responses as they age (Willott, 1986) and have severely depleted levels of melotonin due to a truncation in the enzyme responsible for synthesising it from serotonin (Roseboom et al., 1998). Their DNA is also resistant to modifications due to a high level of *de novo* DNA methylation at many loci (Schumacher et al., 2000). On the other hand they are one of the only inbred strains that perform well in the Morris water maze, a test commonly used for spatial memory (Crawley et al., 1997). Furthermore, some inbred strains also display anatomical abnormalities that may affect behaviour. For example, 129 substrains of mice carry the pink-eyed dilution allele, which is associated with deficits in visual acuity (Brilliant et al., 1994; Crawley et al., 1997) and often show hypoplasia of the corpus callosum (Balogh et al., 1999).

1.1.3.3 *Differences between mouse strains*

Obviously the amount of genetic variation between different mouse strains means that they differ considerably at a molecular and cellular level. These differences in turn give rise to a specific behavioural phenotype for each strain. There have been many studies comparing the behavioural phenotypes of the different inbred and outbred strains.

These include open field behaviour, learning paradigms, drug-induced behaviour, and reproductive and parental behaviours (Crawley et al., 1997). Table 1.1 summarises some of these findings for three different inbred strains. As mentioned in the previous section, the water maze tests spatial learning and B6 mice are the only inbred strain to show robust learning in it. However, in another learning-related phenomenon, contextual fear conditioning, many more strains show a good response implying less strain-dependence for this behaviour (Owen et al., 1997). Acoustic and tactile startle reflexes are another parameter that show variation between strains (Logue et al., 1997; Paylor and Crawley, 1997). The startle reflex is highly conserved between all mammals and can be modulated by salient emotional and cognitive stimuli. It is believed to be involved in sensorimotor gating and the pathways mediating it may be affected in human disease states including schizophrenia. Prepulse inhibition, a related response involving the suppression of the startle reflex by a weaker prestimulus also shows variation between strains (Logue et al., 1997; Paylor and Crawley, 1997). The light/dark paradigm is a method of assessing anxiety-like behaviour and drugs that may affect it. It will be discussed in great detail elsewhere in this thesis. In the test an increased number of transitions between compartments after benzodiazepine administration indicates an anxiogenic effect of the drug. Analysis of several inbred strains in this paradigm showed that there were marked differences between many strains in their responses both at baseline and after diazepam (Crawley and Davis, 1982; Mathis et al., 1994). Drugs of abuse also have a wide range of effects on the different strains. As such, drugs, including ethanol, nicotine, psychostimulants, opiates and the antipsychotics, affect different mice in different ways. For example, the opiates have been studied in detail in the different strains for their effects on different kinds of behaviours including analgesia, opiate withdrawal, morphine preference drinking and hyperlocomotion (Mogil et al., 1996).

With the preponderance of studies conducted in genetically engineered mice, a proper understanding how each strain performs on different tasks is essential to the designing of a good experiment and breeding strategy (Crawley et al., 1997), as will be

Table 1.1: Comparison of different mouse strains. Performance of three inbred strains in behaviours relating to complex learning, startle responses, anxiety and drug-responsiveness. More information and references are given in the text. AU, arbitrary unit. Adapted from Crawley et al. (1997).

Behaviour	C57BL/6	129/sv	DBA/2J
Morris water maze	Good	Poor	Poor
Fear conditioning	Good	Good	Good
Startle reflex (tactile; AUs)	1306 ± 140	425 ± 85	705 ± 138
Startle reflex (acoustic; AUs)	1317 ± 121	186 ± 33	440 ± 91
Pre-pulse inhibition (AUs)	31 ± 8	70 ± 5	48 ± 9
Light—dark transitions	49 ± 3	N/A	21 ± 2
...after diazepam	91 ± 11	N/A	27 ± 7
Morphine-induced locomotion	High	Low	Low

discussed in more detail below.

1.2 *Genome engineering in the mouse*

1.2.1 *Creation of a "knockout"*

There are various methods used to achieve different forms of genome engineering in the mouse. One of the most powerful techniques uses site-specific homologous recombination to produce mutations targeted to the gene of interest. A targeting vector is made comprising of a selection marker flanked by the DNA sequence surrounding the gene of interest. Information about the targeting vector used to create the $NK1^{-/-}$ mouse is given in Section 2.1.2 in the following chapter. This construct will then be transferred to embryonic stem (ES) cells via electroporation. Inclusion of the selection marker in the construct allows cells which have incorporated the vector to be selected for and disrupts transcription of the targeted gene preventing its expression.

Blastocysts are injected with ES cells containing the targeted mutation to produce chimeric mice. Both the blastocysts and ES cells are usually derived from 129 inbred mouse lines as these are most commonly available as pluripotent cell lines. These chimeric mice are then mated with B6 mice. B6 mice are used here as a convenience (i) because they are good breeders and (ii) because the vast majority of laboratory work is conducted using them. If the mutation has been taken up by the germ cells in the chimeric mouse, this cross will produce mice that are heterozygous for the mutated allele. These mice can be detected via techniques such as Southern blotting or polymerase chain reaction (PCR). A further intragenerational cross should produce mice that are both homozygous for either the mutation or the wildtype allele, or heterozygotes (Jockusch et al., 2004; Glaser et al., 2005).

1.2.2 Flanking genes and breeding strategies

Several issues arise from this particular strategy, however. Firstly, the ES cells that traditionally have been used have been derived from 129 mouse lines. This is mainly because they possess particular germline properties which make such genome engineering possible (Jockusch et al., 2004). However, 129 substrains show a great deal of variation between each other and so can have a complex genetic background to begin with. Furthermore, some studies have shown certain ES cell lines to be polymorphic at several loci meaning they cannot have been derived from an inbred line (Simpson et al., 1997). Random segregation of these alleles can therefore lead to a badly defined genetic background. Another key problem is caused by flanking genes. B6 mice are used to breed with the chimeras to generate F1 heterozygotes and subsequently F2 homozygotes. During homologous recombination the closer two alleles are to each other the higher the likelihood that they will be inherited together. This means that the area surrounding the mutated locus is more likely to be derived from the original 129 ES cells whereas in the wildtype allele this region is more likely to originate from the B6 breeders. Differences between the genotypes seen at this F2 stage

have a high likelihood of deriving from a variation between these two background strains rather than from the chosen mutation (Gerlai, 1996). As the 129 and B6 strains, particularly, have been shown to differ on many behaviours as illustrated in table 1.1, it is especially necessary to eliminate these linkage problems. This is commonly achieved by either backcrossing the mutation onto an inbred strain, usually B6, or the more rapid technique of using speed congenics (Jockusch et al., 2004). However, as neither of these can ever truly eliminate the differences in flanking regions other methods have been suggested. At the Banbury Conference in 1997 a group of scientists working in this field met to discuss a series of recommendations to deal with these problems (Silva et al., 1997). These included the establishment of two congenic lines using different genetic backgrounds. Their F1 progeny can then be used as experimental animals.

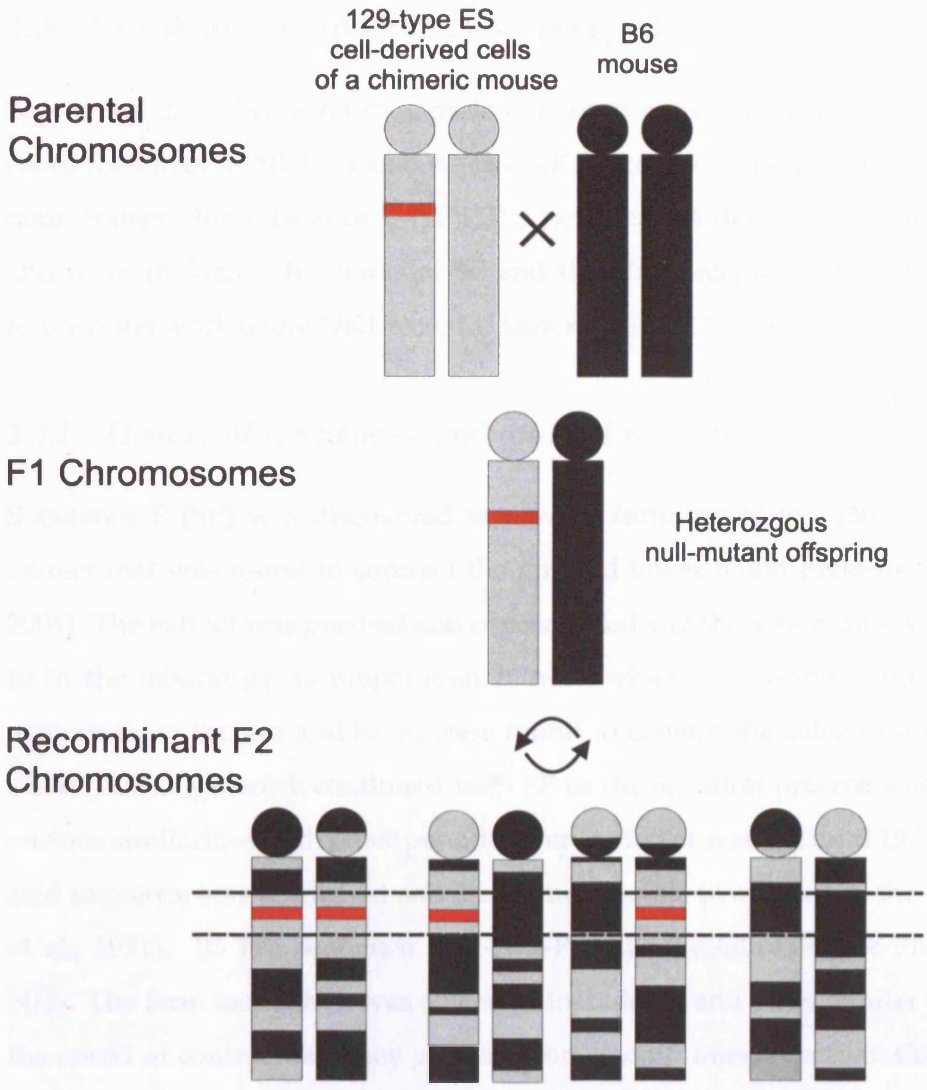

Figure 1.1: Chromosomal constitution of mice generated by gene targeting. Germ-line transmitting chimeric mice from 129-derived ES cells (grey) containing a mutation (red) in one allele are crossed with B6 mice (black) and give rise to an F1 population a proportion of which will be heterozygous for the mutation. Breeding these mice together, by Mendelian laws, creates homozygous wildtype, homozygous mutant and heterozygous mice. Crossover processes during gametogenesis mean that the area surrounding the mutated locus (dashed lines) will be derived from the 129 parental strain whereas the area surrounding the wildtype locus will come from the B6 strain. This linkage disequilibrium and its consequences is explained in more detail in the text. Adapted from Gerlai (1996).

1.3 Substance P and the NK1 receptor

This thesis describes a set of experiments examining how genetic background influences the effect of NK1 knockout. The NK1 receptor is the preferred receptor for the neurotransmitter substance P (SP). The next section details the history, distribution and main biological functions of SP and the NK1 receptor with particular reference to previous work using NK1 receptor knockout ($NK1^{-/-}$) mice.

1.3.1 History of substance P and the NK1 receptor

Substance P (SP) was discovered somewhat fortuitously in 1930 from an intestinal extract that was found to contract the gut and lower blood pressure (Lembeck et al., 2004). The extract was purified and concentrated and the active substance was referred to in the laboratory as preparation P, from which its current name derives. Only extracts from the gut and brain were found to contain the substance (Lembeck et al., 2004). Although work continued with SP as the isolation process was improved and various similarities with other peptides were noted, it was not until 1971 that the amino acid sequence was published and it became possible to synthesize the peptide (Chang et al., 1971). Its full sequence is H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2. The term tachykinin was coined to include SP and other similar peptides due to the speed of contractions they produced on smooth muscle preparations. Neurokinin A and neurokinin B were discovered as new members of this family as well as several non-mammalian tachykinins. In the 1980s the gene and precursor protein to SP were isolated, as well as the preferred receptors: NK1 for SP and NK2 and NK3 for neurokinin A and neurokinin B, respectively (Lembeck et al., 2004).

1.3.2 Biosynthesis of SP

The gene that synthesises the precursor to SP is named preprotachykinin A (*ppt-a* or *TAC1*) with its product similarly named preprotachykinin A (PPT-A). Differential

transcription and/or splicing mean that the same gene is also responsible for producing NKA, neuropeptide K and neuropeptide γ (Conlon and Holzer, 2004). As well as its role in the biosynthesis of SP, recent work has shown PPT-A to contribute to the regulated trafficking of δ-opioid receptors into large dense core vesicles in the spinal cord (Guan et al., 2005). This may have implications for situations in which there is either a mismatch of agonist to receptor as mentioned below or explain differences between the PPT-A$^{-/-}$ and the NK1$^{-/-}$ mice.

1.3.3 Distribution

Several studies have used immunocytochemistry to map the distribution of SP and the NK1 receptor. SP and the NK1 receptor are expressed at high levels both in the gut and in various regions of the central and peripheral nervous system (CNS; PNS). Here, the focus will be on the distribution in the CNS. Distribution patterns show a high degree of similarity between different species and in most cases the receptor expression matches with the agonist distribution, although not in all.

SP is mainly a subcortical peptide and only very low expression of the peptide and the NK1 receptor have been noted in the cortex or hippocampus (Gadd, 2003; Nakaya et al., 1994). The striatum, both the caudate putamen (CPu) and the nucleus accumbens (NAcc), show expression of SP and the NK1 receptor (Penny et al., 1986; Nakaya et al., 1994). SP is found in the dynorphin-positive, GABAergic medium spiny neurons which project to the substantia nigra, globus pallidus and ventral pallidum as well as back onto the cholinergic interneurons within the striatum (Anderson and Reiner, 1990; Napier et al., 1995). The NK1 receptor is found densely expressed on the cell bodies and dendrites of these cholinergic cells and release of SP in the striatum causes an increase in their firing and release of acetyl choline and dopamine.

The medial and central nuclei of the amygdala show expression of SP and the NK1 receptor is expressed strongly in the central, basomedial and medial nuclei as well as some of the amygdala's accessory regions (Emson et al., 1978; Nakaya et al., 1994).

The hypothalamus contains both NK1 receptor and SP immunoreactivity throughout where they are believed to cause release of hormones from the pituitary (Aronin et al., 1986).

The habenular nucleus possesses some of the strongest NK1 receptor expression in the brain, particularly its medial division. This region also sends SP-containing fibres to the ventral tegmental area (VTA) which may be related to the control of locomotor activity, learning and reward processes (Emson et al., 1977).

Paradoxically, the area of the brain with the highest amount of SP is the substantia nigra, a region that seems to have very little NK1 receptor expression (Davies and Dray, 1976; Nakaya et al., 1994; Gadd, 2003).

The periaqueductal gray (PAG) contains some NK1 expression while the dorsal raphe nucleus (DRN), which gives rise to the major serotonergic projections shows well-defined cell bodies (Halliday et al., 1988; Nakaya et al., 1994; Santarelli et al., 2001). Similarly, the parabrachial nucleus (PbN) and the locus coeruleus (LC), which gives rise to the major noradrenergic projections, also show high NK1 expression (Guyenet and Aghajanian, 1977; Nakaya et al., 1994; Santarelli et al., 2001) and are innervated by SP containing fibres (Halliday et al., 1988).

Figure 1.2 shows examples of NK1 receptor staining in a number of these brain regions. The relevance of these expression patterns will be referred to as function is discussed later in this chapter.

1.3.4 Signalling via the NK1 receptor

The NK1 receptor is a member of the G-protein coupled receptor (GPCR) superfamily and as such contains 7 transmembrane domains with a ligand binding site at the extracellular N-terminus. The NK1 receptor signals via the $G_{q/11}$, $G_{\alpha s}$ and $G_{\alpha o}$ proteins which activate phospholipase Cβ (PLCβ) and in turn stimulate the formation of inositol-3-phosphate (IP_3) and diacyl glycerol (DAG) (Taylor et al., 1986; Sugiya et al., 1987; Nakajima et al., 1992; Takeda et al., 1992; Seabrook and Fong, 1993;

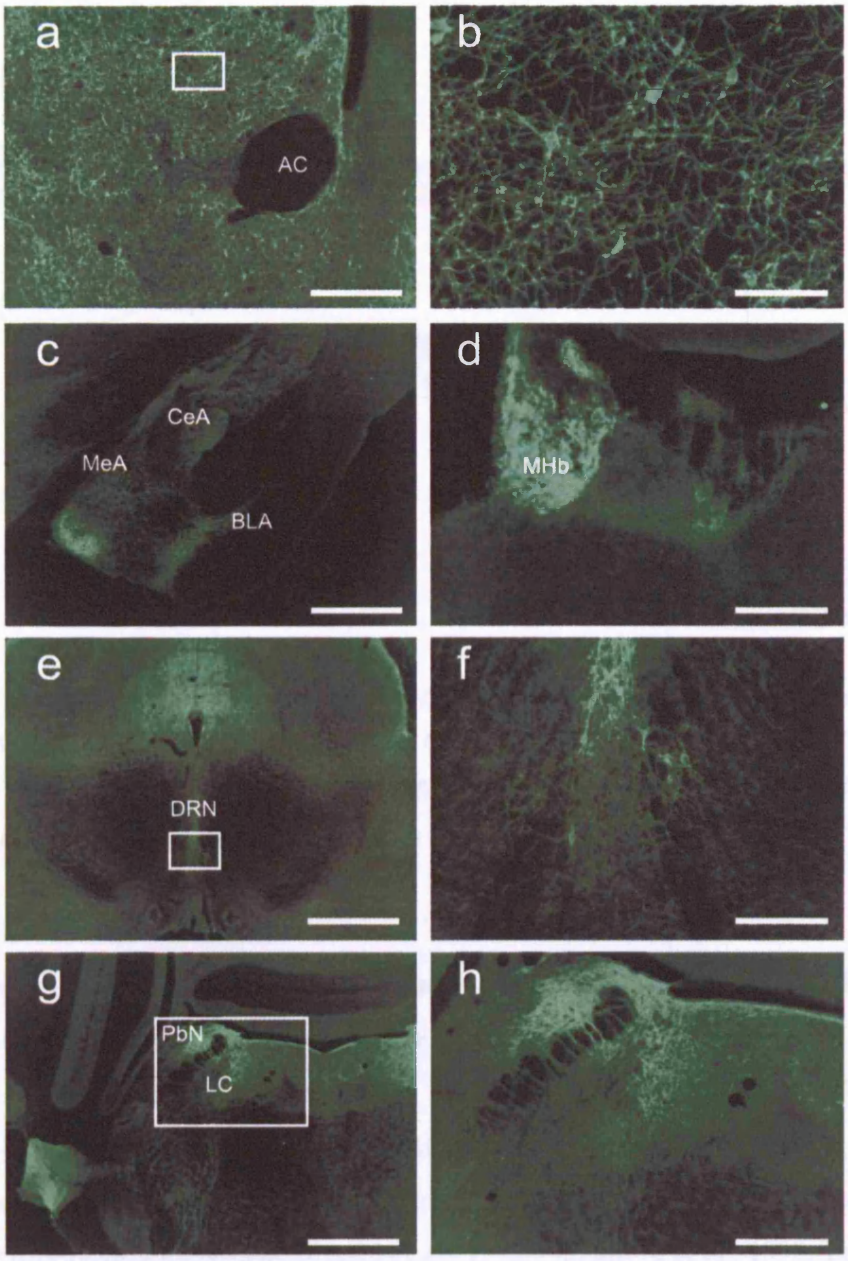

Figure 1.2: NK1 receptor expression in the mouse brain. Panels show immunostaining in the striatum (a,b), amygdala (c), medial habenular nucleus (MHb) (d), periaqueductal grey (e), dorsal raphe nucleus (DRN) (f) and parabrachial nucleus (PbN) and locus coeruleus (LC) (g,h). The immunocytochemical methods used are described in chapter 2 and the appendix. Scale bars, a, h: 250 μm; b, d, f: 100 μm; c, e, g: 500 μm. AC, anterior commissure; CeA, central nucleus of the amygdala; MeA, medial nucleus of the amygdala; BLA, basolateral nucleus of the amygdala.

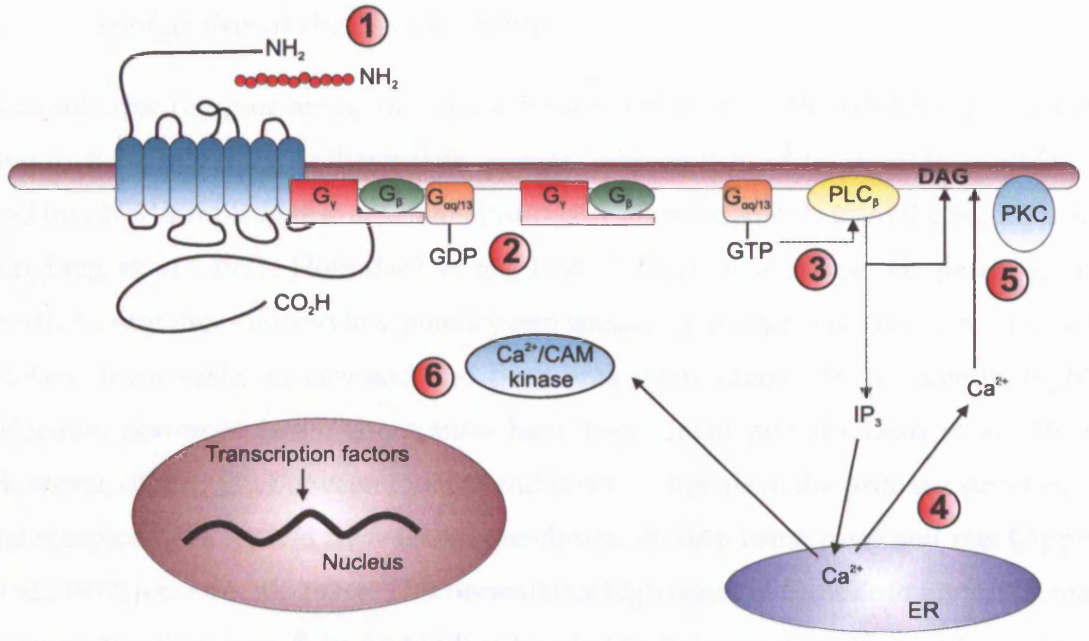

Figure 1.3: The NK1 receptor and its signaling pathway. 1, Substance P binds to the G-protein-coupled NK1 receptor; 2, Receptor-mediated GDP/GTP exchange; 3, $G_{\alpha q/13}$-GTP activation of PLC_β; 4, IP_3-gated intracellular Ca^{2+} mobilisation; 5, PKC activation; 6 Ca^{2+}/CAM kinase activation. Adapted from Bremner et al. (2001)

Macdonald et al., 1996; Roush and Kwatra, 1998). The increase in IP_3 induces the release of calcium from intracellular stores resulting in activation of kinases including calcium-calmodulin-dependent kinase (Merritt and Rink, 1987; Womack et al., 1988). The formation of DAG activates protein kinase C (PKC). Activation of the receptor also activates adenylyl cyclase leading to the accumulation of cyclic adenosine monophosphate (Mitsuhashi et al., 1992; Nakajima et al., 1992; Takeda et al., 1992) and stimulates arachidonic acid production via phospholipase A_2 (Garcia et al., 1994). Together these processes lead to a slow excitatory potential by increasing sodium conductance and decreasing potassium conductance (Nicoll, 1978; Nowak and Macdonald, 1982; Stanfield et al., 1985; Shen and North, 1992) and can also lead to modulation of gene transcription. This signalling pathway is illustrated in figure 1.3.

1.3.5 *Antagonists at the NK1 receptor*

A number of receptor antagonists have been developed for the NK1 receptor partly due to their potential as therapeutic agents. The earliest of these were peptidergic and involved simple amino acid substitutions or rearrangements from the SP sequence (Engberg et al., 1981; Holmdahl et al., 1981; Folkers et al., 1984; Hagiwara et al., 1994). Mostly they showed low potency and specificity, a degree of neurotoxicity, were broken down easily *in vivo* and had poor brain penetrance. More recently, highly selective, non-peptidergic antagonists have been developed (McLean et al., 1996). However, differences between rodents and other mammals in the primary structure of the receptor have limited their use in pre-clinical models using mice and rats (Appell et al., 1992; Jensen et al., 1994). This necessitates high doses in these animals which may compromise their specificity by binding to other tachykinin or neuropeptide receptors or interfering with calcium channel function (Schmidt et al., 1992; Guard et al., 1993). Often, gerbils or guinea pigs, which have a receptor structure far closer to humans, have had to be used instead. However, despite these limitations many of the studies have contributed useful data to the field and have been supported by later work using different approaches. These will be reviewed below where appropriate.

1.3.6 *The NK1$^{-/-}$ mouse*

The use of genomic engineering to create mice lacking a functional NK1 receptor (NK1$^{-/-}$ mice) has greatly aided study in this area. Three separate lines of NK1$^{-/-}$ mouse have been created using homologous recombination in different laboratories (Bozic et al., 1996; De Felipe et al., 1998; Santarelli et al., 2001). In general similar findings have been produced although where there are discrepancies these are likely due to differences in background strain or experimental procedure. The mice used in this thesis derive from those produced by C. De Felipe and A.H. Smith and are described in Section 2.1.2 in the following chapter (De Felipe et al., 1998).

1.4 Functions of SP and the NK1 receptor

The next few sections will consider the functions of SP and the NK1 receptor with reference to a number of behaviours.

1.4.1 Nociception

The most widely studied physiological role of SP has been its involvement in processing painful stimuli. The next section briefly describes the neuroanatomy of nociception, the animal models used to study it and the function of SP and the NK1 receptor within this system.

1.4.1.1 The neuroanatomy of pain

Pain is an experience consisting of both a sensory and an emotional component (International Association for the Society of Pain). Under non-pathological conditions it requires peripheral activation of non-myelinated afferents known as C-fibres and thinly myelinated Aδ fibres. Their cell bodies are located in dorsal root ganglia (DRGs) and their axons project to the periphery, where their terminals detect noxious stimuli, as well as centrally where they synapse on cells in the dorsal horn of the spinal cord (Todd and Koerber, 2006). The fibres are polymodal and will respond to thermal, chemical or mechanical stimuli. From the dorsal horn ascending projections relay the information to higher brain areas such as the somatosensory cortex, the anterior cingulate cortex and the amygdala, which are important for either the sensory or affective responses (Hunt and Mantyh, 2001). Projections to areas of the hind brain such as the rostroventral medulla (RVM) may also be important for mediating modulatory influences (Mantyh and Hunt, 2004). Under pathological conditions parts of this circuitry may become activated by either normally neutral stimuli or in the absence of peripheral stimulation. Neuropathic or inflammatory diseases can cause this and often arise from physical insults to the nervous system (Campbell and Meyer, 2006).

1.4.1.2 *Animal pain models*

Most of the animal pain models use either chemical or surgical methods to mimic these long lasting pathologies allowing the underlying alterations to be studied. Surgically, ligation, constriction or axotomy of a nerve will produce central changes and a heightened sensitivity to pain. Chemically, peripheral administration of noxious substances such as capsaicin, formalin, or carrageenan will also result in persistent changes in pain sensation (Wang and Wang, 2003; Vierck, 2006).

1.4.1.3 *The role of SP and the NK1 receptor in pain*

The role of SP in processing nociceptive stimuli has been studied for many years. SP was originally hypothesised to be the primary neurotransmitter responsible for transmitting information about painful stimuli. This was prompted by the knowledge that SP was released in the spinal cord by painful manipulations (Randic and Miletic, 1977). Additionally, SP is localised within a small subset of small diameter DRG cell bodies and terminal fields of the nociceptive primary afferents (Li and Zhao, 1998) and the NK1 receptor is found on the laminar I, III and V cells of the dorsal horn of the spinal cord that project to the brain (Nakaya et al., 1994). Furthermore, intrathecal administration of SP causes biting and scratching of the abdomen (Gamse and Saria, 1986) and painful stimuli increase mRNA for both PPT-1A and the NK1 receptor in DRG cell bodies (Noguchi et al., 1988; McCarson, 1999). However, a role for SP or the NK1 receptor in the detection or modulation of acute painful stimuli has not been demonstrated. Although antagonists will reduce chronic inflammatory or neuropathic pain in many models they have no effect on acute pain. This is supported by work using the $NK1^{-/-}$ mouse in which the second, but not the first, phase of the formalin test is attenuated (De Felipe et al., 1998). A reduction in secondary hyperalgesia produced by intraplantar capsaicin administration (Laird et al., 2000; Mansikka et al., 2000) and Mycobacterium tuberculosus (Kidd et al., 2003) have also been found as well as a decrease in intensity coding of painful stimuli (Suzuki et al., 2002). Distribution of

the ascending projections from the NK1-positive cells in laminar I have also hinted that SP may play a role in processing of the affective, rather than the sensory, components of pain (Suzuki et al., 2005). Current work also suggests that these cells form part of a spino-bulbo-spinal loop that produces descending modulation via connections to the PbN and the PAG and from the RVM (Millan, 2002; Suzuki et al., 2002). Together these results imply a role for SP in mediating long lasting pain states.

1.4.2 Drug abuse and addiction

SP and the NK1 receptor have been implicated in drug abuse and addiction, especially to the opiates. Here, the neurobiology and anatomy of drug addiction will be described as well as the various animal models used.

1.4.2.1 Anatomy and neural bases of drug addiction

The neural substrate common to almost all drugs of abuse is the dopaminergic projection that runs from the ventral tegmental area (VTA) of the hindbrain and terminates in the ventral striatum or nucleus accumbens (NAcc) (Wise, 1998; Nestler, 2001). This pathway is normally activated by natural rewards and reinforcers and is important for learning motivated behaviours (Berke and Hyman, 2000). Under pathological conditions of addiction, however, it can become strongly stimulated by drugs or drug-associated stimuli. Other areas such as the amygdala, prefrontal cortex and hippocampus also have a modulatory influence as do the monoaminergic projection areas, the LC and DRN, which supply noradrenaline (NA) and serotonin (5-HT), respectively, to the forebrain (Everitt et al., 1991; Carlezon and Wise, 1996; Nestler and Aghajanian, 1997; Nestler, 2001).

1.4.2.2 *Models of drug addiction*

There are a number of models used to test various aspects of drug abuse: conditioned place preference (CPP), behavioural sensitisation, self administration, and tolerance and physical dependence. CPP refers to the pairing of a drug with a particular environmental context and is thought to test the rewarding properties of a drug (Bardo and Bevins, 2000). Behavioural sensitisation is the development of an augmented response, usually locomotor, after repeated administration of a drug and is believed to model the craving associated with drug abuse (Stewart and Badiani, 1993; Robinson and Berridge, 2003). Thirdly, in self-administration studies animals are required to perform an operant response, normally a lever press, to obtain an infusion of drug. This is thought to test the reinforcing properties of a drug (Self, 1998). Finally, tolerance to a drug, resulting from its repeated administration, and physical withdrawal, precipitated by the cessation of drug treatment, can also be produced in animals (Nestler and Aghajanian, 1997; Harrison et al., 1998).

1.4.2.3 *SP and the NK1 receptor in drug addiction*

A potential role for SP in drug addiction was first suggested by the finding that both systemic and local injection of SP into the nucleus basalis magnocellularis induced CPP in rats (Hasenohrl et al., 1998, 2000). Injection of SP intracerebroventricularly (i.c.v.) or local injection into different areas of the midbrain and striatum have also been shown to mimic some features of drug administration such as stimulated locomotor activity and rats will also self-administer SP into the striatum (Elliott et al., 1992; Krappmann et al., 1994).

The $NK1^{-/-}$ mouse fails to develop CPP or behavioural sensitisation to opiates and will not self-administer morphine or heroin (Murtra et al., 2000; Ripley et al., 2002). Antagonism of the NK1 receptor also reduces opiate-induced locomotion (Placenza et al., 2006). In contrast, all these drug-associated behaviours develop with cocaine and, additionally, CPP to natural rewards such as food is still present (Murtra et al.,

2000). Furthermore, although opiate withdrawal is attenuated in the $NK1^{-/-}$ mouse the analgesic properties of morphine are unaffected (De Felipe et al., 1998).

1.4.3 Affective disorders

Affective disorder is a broad term encompassing major depression and anxiety as well as a range of other psychiatric complaints such as posttraumatic stress disorder (PTSD), obsessive compulsive disorder (OCD) and attention deficit hyperactivity disorder (ADHD). These are growing problems around the world and represent a massive financial burden (Murray and Lopez, 1996). A number of medicines do exist which, when used in conjunction with counselling or cognitive therapy, can produce dramatic results. However, in many patients the current treatments have little effect or succeed only in preventing some of the more severe symptoms e.g. suicidal tendencies, without providing a cure (Ebmeier et al., 2006). The NK1 receptor has been implicated in the pathopsychology of some of these disorders, most notably depression and anxiety (Rupniak et al., 2000, 2001; Santarelli et al., 2001, 2002; Gadd, 2003). The next section reviews the neural bases believed to underlie these conditions before considering how SP and the NK1 receptor may be involved in mediating them.

1.4.3.1 The neurological basis of affective disorders

Most of what is known about the pathophysiology of depression and anxiety is due to the medicines we have to treat them. In depression these are the antidepressants, classically the monoamine oxidase inhibitors (MAOs) and the tricyclic antidepressants (TCAs), and in anxiety the benzodiazepines (BZs) (Ebmeier et al., 2006).

The classic antidepressants were discovered by serendipitious clinical findings in the 1950s. Research into depression since then has mainly focused on investigating these drugs' targets rather than examining the underlying pathopsychology (Berton and Nestler, 2006). These drugs, as well as the more modern classes of antidepressant, all work by increasing monoaminergic tone in the brain. Accordingly, distur-

bances of the monoaminergic system comprising of noradrenaline (NA), serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) seem to be an important factor in depression (Nestler et al., 2002). MAOs inhibit the breakdown of these compounds and selective serotonin reuptake inhibitors (SSRIs) and noradrenaline reuptake inhibitors (NRIs) prevent the reuptake of 5-HT and NA into the synaptic terminals, respectively. TCAs are also believed to inhibit monoamine uptake although their exact mechanism of action is less well understood (Manji et al., 2001; Nestler et al., 2002).

However, although monoamine levels are increased by antidepressants almost immediately, a behavioural improvement in the patient is often not seen for a number of weeks. Furthermore, once it has occurred this alleviation of symptoms may persist long after cessation of treatment (Nestler et al., 2002). These points argue against a simplistic explanation and imply that a secondary event must take place before the depression is alleviated.

A popular view is that increased monoaminergic tone may be the first step in a complicated cascade of molecular changes that lead to gene regulation. One candidate molecule is the 5-HT_{1A} autoreceptor. Activation of the receptor in the dorsal raphe nucleus (DRN) inhibits firing and so decreases serotonergic tone in the forebrain. Prolonged antidepressant treatment has been shown to functionally desensitise the receptor, thereby increasing serotonin levels in the brain (Le Poul et al., 2000).

Another view is that hippocampal neurogenesis may be crucial for the alleviation of depression. Several observations have linked disturbances in neurogenesis to the pathophysiology of depression. Cognitive deficits in depressive states have implicated impaired hippocampal function (Austin et al., 2001). Additionally, patients with depression have smaller hippocampal volumes when using structural magnetic resonance imaging (MRI) (Sheline et al., 1996; Bremner et al., 2000). In animals, chronic treatment with most of the major antidepressants or with electroconvulsive shock therapy (ECT) increases neurogenesis (Madsen et al., 2000; Malberg et al., 2000; Santarelli et al., 2003). The neurogenesis caused by antidepressants is required for their behavioural effects (Malberg et al., 2000; Santarelli et al., 2003). Antidepressant

treatment also upregulates hippocampal brain-derived neurotrophic factor (BDNF) (Coppell et al., 2003). BDNF and other neurotrophins have been implicated in the pathophysiology or depression and the actions of antidepressants (Duman and Monteggia, 2006). Stress, which is also known to be a major precipitating factor in depression in humans and animals, decreases neurogenesis as well as reducing BDNF expression (Duman and Monteggia, 2006; Tsankova et al., 2006). High corticosterone levels have also been shown to decrease neurogenesis *in vitro* and *in vivo*, in rodents and primates (Gould and Tanapat, 1999; Wong and Herbert, 2004). Early life stress also decreases neurogenesis in adulthood (Karten et al., 2005). Furthermore, subjecting animals to an inescapable stressor decreases neurogenesis and increases behavioural depressive symptoms, which are reversed by antidepressant treatment (Malberg and Duman, 2003). Finally, the time taken for neurons to be produced and reach maturity closely matches that observed in the clinic for depressive symptoms to alleviate after the start of antidepressant treatment (Sapolsky, 2004). However, there is still work to be done to prove the necessity for neurogenesis in human treatment and to elucidate whether it is a causative factor or an epiphenomenon.

Another possible explanation for the lag between treatment and effect has suggested that antidepressants might exert their effects through changes in chromatin structure. Using an animal model Tsankova et al. (2006) showed increased DNA methylation at BDNF promoter regions after chronic psychosocial stress in mice. This methylation seems to be a permanent modification and results in a reduction in hippocampal BDNF. Chronic, but not acute, treatment with antidepressants resulted in acetylation of histone proteins associated with BDNF promoter regions. Although the methylation of these regions was not reversed, an increase in BDNF expression was observed and alleviation of the depressive-like symptoms was seen in their behavioural model.

Clearly, all of these different processes are likely to be important in the treatment of depression and will contribute to the efficacy of antidepressants. It is of importance now to elucidate which are the most crucial in determining the speed of treatment

effect and the length of remission as well as which ones contribute to the pathogenesis of depression. Patients presenting for depression have often been suffering for weeks, months or even years before seeking help. Consequently, clinical findings such as anatomical changes in brain regions may be an effect rather than a cause of the depression (Ebmeier et al., 2006). After treatment this problem becomes greater as the effect of the particular treatment regimen also needs to be taken into account as well. With studies using *post mortem* tissue these problems are greatest as the sufferer is likely to have experienced many bouts of depression and accordingly many different treatment regimes over a period of years (Rajkowska, 2000). The cause of death often may be related to the depression, suicide or drug overdose being very common (Purselle and Nemeroff, 2003; Ebmeier et al., 2006). Disentangling all these factors therefore becomes very difficult.

1.4.3.2 Genetics and affective disorders

Over recent years many studies have attempted to find genetic links to depression. Several candidate genes have been identified as particular vulnerability alleles. Of these, a polymorphism in the serotonin transporter has generated the most interest (Anguelova et al., 2003; Lasky-Su et al., 2005). Other key components such as BDNF, catecholamine methyl transferase (COMT) and D-amino acid oxidase activator (DAOA) have also been shown to vary in people who suffer from depression (Craddock and Forty, 2006; Ebmeier et al., 2006).

1.4.3.3 Stress, the HPA axis and affective disorders

Another common feature of affective disorders that may contribute to their pathogenesis is hyperactivity of the hypothalamic-pituitary-adrenal axis (HPA axis; see section 5.1.1 in Chapter 5). Many depressed patients have an elevated and flattened circadian rhythm of corticosterone production (Holsboer, 2000; Gillespie and Nemeroff, 2005). In animals in which this rhythm has been induced experimentally, negative

feedback that acts to terminate response to a stressor via the glucocorticoid receptor is impaired. Similarly, patients with depression subjected to the dexamethasone repression test also show this impairment (Modell et al., 1997). Depressed patients also show a blunting of the ACTH response to CRF challenge and increased CRF levels in the CSF (O'Keane et al., 2005). Behavioural responses to chronic stress in animal studies show similar features to depression seen in humans, for example, anhedonia (Willner, 1997; de Kloet et al., 2005).

1.4.3.4 Animal models of affective disorders

Preclinical animal models pose a particular problem to those working in the field of affective disorders. Clearly, modelling of a complex psychological disease such as depression in a rodent is a challenge. As well as difficulties in assessing when a rodent is 'depressed' it could even be argued that differences in the complexity of human and animal brains would question the existence of any disease akin to depression in lower mammals (Cryan and Mombereau, 2004). However, as drugs exist, such as the classical antidepressants, which alleviate disease symptoms in human patients and have observable effects in animals, we are able to use these to help validate the models. Although this approach may help to identify new drugs it is likely that these will work via the same mechanisms as established treatments. As such, the optimal treatment solution, that will come only with a full understanding of the disease processes, may remain obscured (Berton and Nestler, 2006).

Despite these caveats there is much preclinical work using animal models of affective disorders and progress is being made in their development and refinement (Lucki, 2001). In depression research the two most widely used models are the forced swim test and the tail suspension test (Porsolt et al., 1977; Cryan and Mombereau, 2004). After being placed in an inescapable situation rodents will eventually appear to give up and become immobile. This cessation of struggling behaviour is known

as learned helplessness or behavioural despair. Many antidepressants administered acutely will decrease this immobility time.

A group of models using chronic stressors have gained popularity more recently. Depending on the precise protocol the stressor may be either physical or psychosocial in nature and the outcome measure may have a physiological readout or a behavioural one, such as decreased social interaction (Willner, 1997). Again, antidepressants have been used to validate these models but, as opposed to the forced swim or tail suspension test, in general, chronic administration is needed for an effect of treatment (Tsankova et al., 2006). Taken together with the fact that stress is supposed to be an important factor in leading to depression, these models appear to have more construct validity and be more ethologically sound than the previous standards.

A related group of models use early life manipulations to induce a depression-like syndrome. Postnatal animals subjected either to an emotional stressor such as prolonged maternal separation or an immunological challenge will develop a number of lifelong behavioural and physiological alterations including anhedonia, decreased social interaction and hyperresponsivity of the HPA axis (Plotsky and Meaney, 1993; Millstein and Holmes, 2006).

As with depression research, difficulties in interpreting animal behaviour in terms of a complex human disease necessitate the use of preclinical models of anxiety that have been validated by the use of drugs known to have an anxiolytic or anxiogenic effect in humans. Generally, benzodiazepines are used for this purpose (Belzung and Griebel, 2001). The most commonly used model is the elevated plus maze (EPM). When given benzodiazepines, rodents will make more entries into the maze's open arms. Any other manipulation — pharmacological, genetic or otherwise — that mimics this effect could be said to be anxiolytic (Lister, 1987). Another commonly used model is the open field test in which increased time spent in the centre of the arena is believed to reflect anxiolytic behaviour. Both of these tests suffer from large differences in sensitivity between strains, testing centres and individual animals. Effects of a particular manipulation on locomotor behaviour may also confound the

results. Additionally, although traditional anxiolytics such as benzodiazepines give consistent results other compounds recently tested such as 5-HT_{1A} receptor agonists (e.g. 8-OH-DPAT, busiprone) have yielded discrepancies (Lee and Rodgers, 1991; Micheau and Van Marrewijk, 1999)

As with the depression models with many of these tests it is highly likely that a single simplistic aspect of anxiety-like behaviour is being examined rather than the complex, multi-factorial disease we know anxiety to be in the human. Equally, most of the traditional tests are unable to differentiate between the anxiolysis induced by different anxiolytic compounds. Clinical findings suggest that these different agents have distinct effects depending on the anxiety disorder being treated. It has been suggested that the spatiotemporal measures normally used are supplemented, if not replaced, with more ethological behaviours such as head-dipping, time spent in flat-back approach or other such risk-assessment behaviours (Rodgers, 1997). Studies using these measures in a modified form of the test have found that they are normally more sensitive to drug effects than the avoidance behaviours. The light/dark exploration box (LDEB), which is described in more detail later in this thesis, is another model in which the spatiotemporal parameters can be complemented by a number of other ethological measures (Bourin and Hascoet, 2003). Similarly, the mouse defense test battery can also be used to provide a comprehensive behavioural profile (Blanchard et al., 1997). As such, models which are capable of providing a more complete profile of a drug's actions spread over a number of different ethological parameters may be of more use in designing new drugs and interpreting their effects.

Models for the other affective disorders also exist which may overlap to a certain extent with those for depression and anxiety. For PTSD the models focus more on the encoding of a single traumatic event by combining measures of an affective behaviour such as anxiolysis with a conditioning paradigm (Siegmund and Wotjak, 2006). In ADHD research, impulsivity and the response to amphetamines are thought to be important features of most models (Granon and Changeux, 2006).

1.4.3.5 *The NK1 receptor and affective disorders*

The NK1 receptor has been implicated in both depression and anxiety. Promising preclinical work has led to NK1 receptor antagonists' introduction in clinical trials as a putative new class of antidepressant (Kramer et al., 2004). Although these have met with varying degrees of success, the preclinical and clinical work leading up to this will now be discussed.

Established antidepressants such as fluoxetine are known to inhibit neonatal vocalisations induced by maternal separation in guinea pigs. Comparable effects have been found using several NK1 receptor antagonists, MK869, L-760735 and L-733060 (Kramer et al., 1998; Rupniak et al., 2000). Additionally, blocking SP release in the amygdala following aversive stimulation prevents vocalisations associated with these stimuli (Ebner et al., 2004). NK1 antagonists also reduce anxiety in the rat and the gerbil in the social interaction test. This work led to the first clinical trials in which MK869 was shown to reduce major depressive disorder with moderately high anxiety (Kramer et al., 2004). However, further work was hampered by differences in NK1 receptor pharmacology between species.

The $NK1^{-/-}$ mouse also shows a very similar profile to that produced by the antagonists and exhibits alterations similar to those observed after chronic antidepressant treatment. Knockout mice show a decrease in neonatal separation-induced vocalisations compared to wildtype and an increase in struggle behaviour in the forced swim test and tail suspension test, effects that are mimicked in the wildtype by administration of an antidepressant such as fluoxetine (Rupniak et al., 2000; Santarelli et al., 2001). Similar findings have been produced after knockout of the PPT-A gene (Bilkei-Gorzo et al., 2002). The $NK1^{-/-}$ mouse is also reported to have reduced aggression in the resident intruder test (De Felipe et al., 1998).

In tests for anxiety-like behaviour there is less consensus of opinion. Some groups have found increased exploratory behaviour in open field, increased open-arm entry in the elevated plus maze (EPM) and a reduction in novelty-suppressed feeding, in

the $NK1^{-/-}$ mouse suggesting an anxiolytic phenotype (Santarelli et al., 2001, 2002). These differences were not reported in the $NK1^{-/-}$ mouse developed by De Felipe et al. (1998); Rupniak et al. (2001). However, reductions in risk-assessment behaviours in the light/dark exploration box imply an anxiolytic profile in this mouse as well (Herpfer et al., 2005; Fisher, 2005). The most parsimonious explanation for these discrepancies is the difference in background strain of the two mice.

As mentioned in the previous section increased hippocampal neurogenesis is thought to be related to antidepressant activity. $NK1^{-/-}$ mice have been shown to have increased neurogenesis in the hippocampus. This increase is at approximately the same level as is caused by antidepressant treatment and is correlated with an upregulation of BDNF. Furthermore, administration of the antidepressants desipramine and paroxetine increased neurogenesis in wildtype but not $NK1^{-/-}$ mice (Morcuende et al., 2003).

Another pathway in which the NK1 receptor may be impacting on affective behaviours is by modulating monoaminergic tone in the nervous system. As already mentioned the NK1 receptor and SP are highly expressed within the LC and the DRN (Froger et al., 2001; Santarelli et al., 2001; Lacoste et al., 2006), the major projections for NA and 5-HT, respectively. Many studies have shown that disruption or antagonism of the NK1 receptor can lead to profound changes in regulation of the monoaminergic systems.

The LC is the main noradrenergic input area to the forebrain. Within the LC almost every neuron expresses the NK1 receptor at a high level and the region is heavily innervated by SP-containing fibres (Halliday et al., 1988; Nakaya et al., 1994; Chen et al., 2000; Santarelli et al., 2001). Application of SP to the LC either *in vivo* in an anaesthetised rat or *in vitro* in a slice preparation increases the firing rate of the majority of cells (Guyenet and Aghajanian, 1977; Cheeseman et al., 1983). Paradoxically, NK1 antagonists have also been shown to increase activity within the LC. L-760735 induces burst-firing of LC neurons (Maubach et al., 2002) whilst L-760735 increases the firing rate of LC cells in the anaesthetised rat (Millan et al., 2001). Furthermore, using microdialysis the NK1 antagonist GR205171 has also been shown to increase

cortical NA efflux in freely-moving rats (Millan et al., 2001) and anaesthetised $NK1^{-/-}$ mice show elevated cortical NA compared to wildtype counterparts (Fisher, 2005). Whilst the NK1 antagonists WIN51,708 and CP-96,345 have no affect on spontaneous firing-rates in the LC, they do attenuate the actions of clonidine, an α2-adrenoceptor antagonist, supporting a role for this autoregulatory receptor in the interaction between NA and the NK1 receptor. Recently it has also been shown that this receptor, which acts to inhibit NA release, is functionally desensitised in the $NK1^{-/-}$ mouse (Fisher, 2005).

The serotonergic system is also modulated by the NK1 receptor. NK1 receptor antagonists and knockout of the NK1 receptor both cause a downregulation and functional desensitisation of inhibitory presynaptic 5-HT_{1A} receptors in the DRN (Froger et al., 2001; Santarelli et al., 2001). This downregulation also occurs following chronic antidepressant treatment. Normally, administration of an SSRI such as fluoxetine results in an initial increase in 5-HT release which is subsequently reduced via its effects at the 5-HT_{1A} receptor (Le Poul et al., 2000). The downregulation/desensitisation of the receptor, caused by blockade or disruption of the NK1 receptor, therefore leads to an enhancement of 5-HT efflux after SSRI challenge with, for example, paroxetine (Froger et al., 2001; Santarelli et al., 2001). As the time course for downregulation/desensitisation of the 5-HT_{1A} receptor seems to follow that required for behavioural effects of antidepressants, this feature is believed to be crucial for their actions (Nestler et al., 2002).

1.4.3.6 The NK1 receptor and stress

SP and the NK1 receptor are also critically involved in many stress-associated behaviours. As such agonists at the NK1 receptor injected centrally cause the appearance of species-specific defensive behavioural changes (Elliott et al., 1992; Bristow and Young, 1994; Rupniak and Williams, 1994; Piot et al., 1995; Rupniak et al., 2000) and cardiovascular responses (Culman and Unger, 1995; Tschope et al., 1995). NK1 receptor antagonists reduce the stress-induced increase in the noradrenergic pathways

that originates in the LC (Hahn and Bannon, 1999). The NK1 receptor seems therefore to have an important role in orchestrating the response, both behavioural and physiological, to external stressors. This is backed up by the finding that $NK1^{-/-}$ mice have reduced stress-induced analgesia and reduced aggression in the resident intruder test (De Felipe et al., 1998).

1.5 Summary and aims

The NK1 receptor and SP are expressed widely throughout the nervous system and contribute to many behaviours including nociception, addiction and affective disorders. Knockout of the receptor has been shown to affect many of these and manipulation of the receptor or neurotransmitter may prove to be an effective therapeutic treatment. Despite the preponderance of studies using genetically modified models, there are few that examine the role of background strain in modulating the effect of a genetic manipulation.

In this thesis I will summarise a series of experiments which examine the influence of genetic background on knockout of the NK1 receptor. I will concentrate on behaviours related to addiction and affective disorders. The results in the different strains will be linked to possible disturbances in the HPA axis and certain components of it will be examined.

Chapter 2

Materials & Methods

2.1 Subjects

2.1.1 Housing

All experiments described in this thesis used mice of between 6 and 16 weeks old. They were housed in the Biological Services Unit, UCL, London 2-6 per cage and maintained on a 12 h light/dark cycle (lights on at 8 a.m.), at a temperature of 21±1 °C and 50 % humidity. Singly-housed mice were never used due to the adverse effects on their behaviour and stress responses. Food (Harlan Teklad TRM Rat/Mouse Diet; Harlan, Bicester, UK) and tap water were available *ad libitum*. All animal procedures were carried out under the UK Animals (Scientific Procedures) Act 1986.

2.1.2 Creation of the NK1-/- mouse

A number of different strains of $NK1^{-/-}$ mice were used in this thesis. In this section the creation of the original knockout mouse will be described first. The crosses used to create the different strains will then be discussed as well as the nomenclature to be used throughout the thesis.

Three different strains of NK1 receptor knockout ($NK1^{-/-}$) mice were used in this thesis. All three strains were derived from those described by De Felipe et al. (1998). Targeted disruption of the gene encoding the NK1 receptor, as illustrated, was used to create the knockout line. A cassette consisting of an internal ribosome entry site

(IRES), the *lacZ* coding sequence and a neomycin resistance gene was inserted at a unique *StuI* site causing disruption of exon 1. These constructs are shown in figure 2.1. Homologous recombination in embryonic stem cells and subsequent mating generated a 129/sv x C57BL/6 mouse line. From this mouse three different strains have been created and used in this thesis. These are illustrated in figure 2.2.

2.1.2.1 MF1 mice

The MF1 mice referred to in this thesis were produced by crossing the homozygous mutant 129/sv x C57BL/6 created by De Felipe et al. (1998) once onto the MF1 background (Harlan). This procedure allowed rapid dilution of the 129/sv component of the mice which has been shown to perform poorly on a number of behavioural tests and diminished the influence of flanking genes.

2.1.2.2 B6 mice

The B6 mice referred to in this thesis were produced by crossing the homozygous mutant 129/sv x C57BL/6 onto the C57BL/6 background over 10 generations. This breeding procedure further diluted out the 129/sv background and removed the confounding effect of flanking genes. Additionally, as C57BL/6 mice perform well in cognitive tests it improved the suitability of the knockout mouse for this area of study.

2.1.2.3 B6:129 mice

The B6:129 mice referred to in this thesis were produced by crossing the homozygous B6 mutant described above once onto the 129/sv background (Harlan). The reintroduction of the 129/sv component was undertaken for reasons given elsewhere in this thesis.

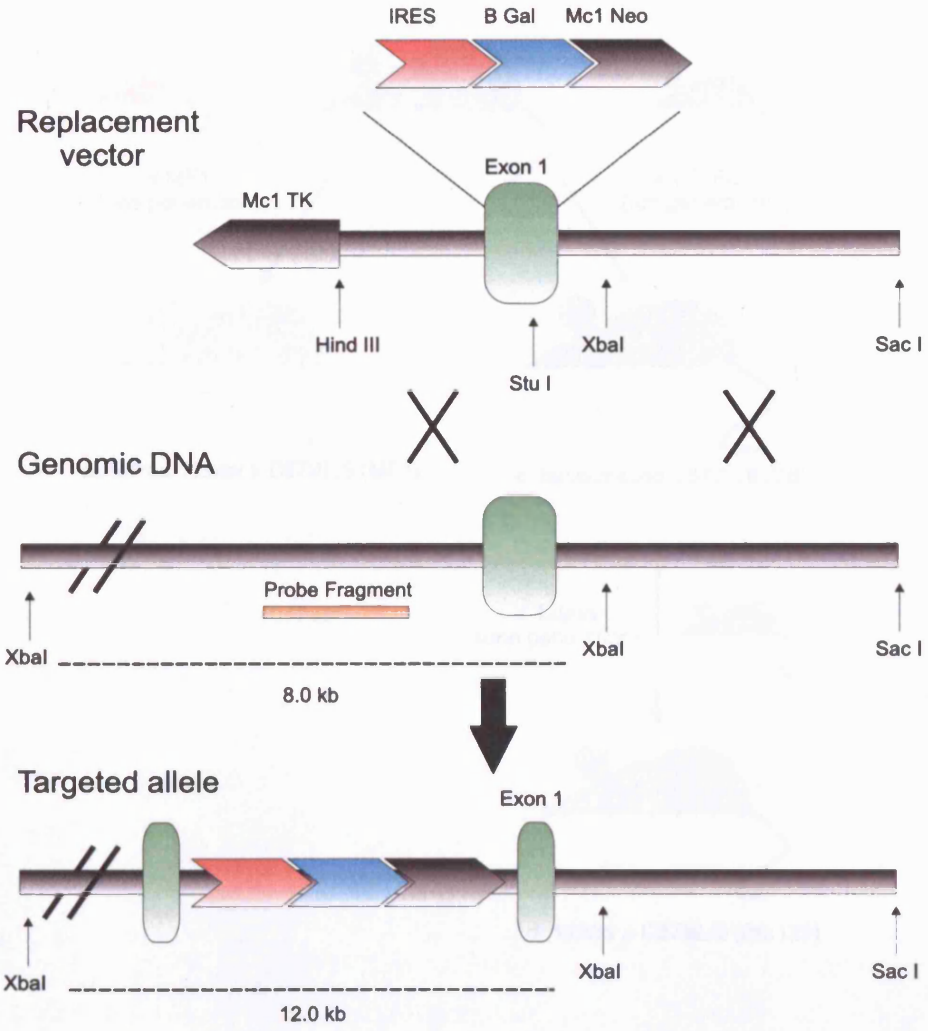

Figure 2.1: The NK1$^{-/-}$ construct used by De Felipe et al. (1998). Using homologous recombination, the NK1 gene was disrupted by insertion of the targeting (replacement) vector into the region of the wildtype NK1 locus containing exon 1. The 5' probe fragment used for Southern blot confirmation and the sizes of predicted restriction fragments following *XbaI* digestion are also shown. B Gal, *lacZ* coding region; IRES, internal ribosome entry site; MC1 MC1 promoter; Neo, neomycin resistance gene; TK, Herpes simplex virus thymidine kinase gene. Small arrows indicate restriction sites. Adapted from Gadd (2003).

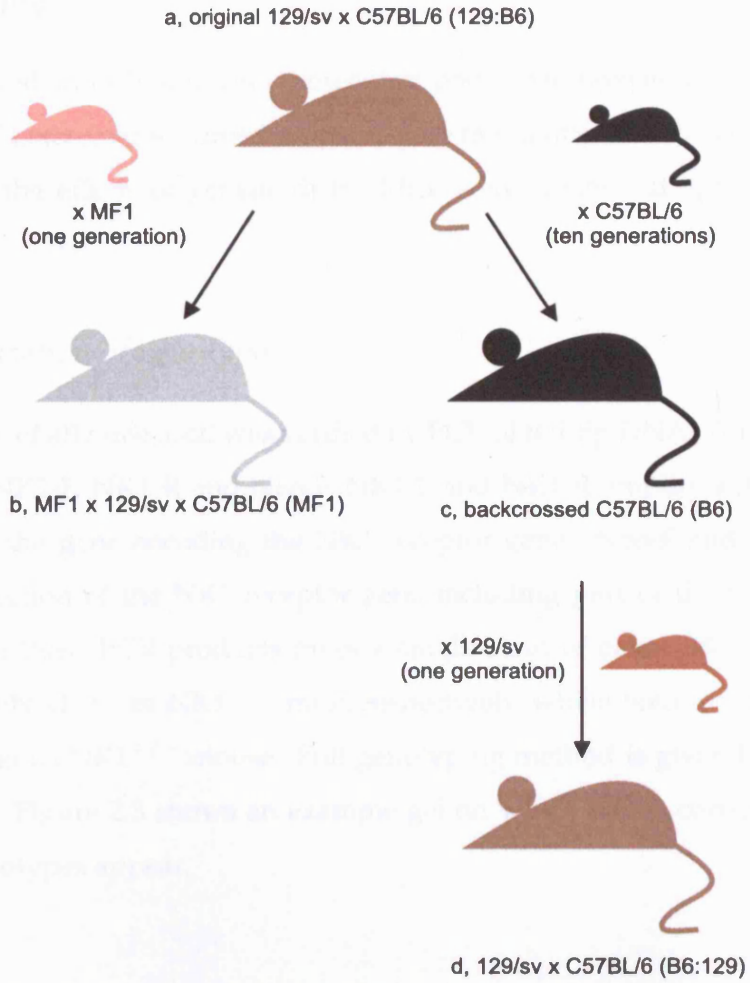

Figure 2.2: The different strains of NK1$^{-/-}$ mouse. The original 129/sv x C57BL/6 (129:B6; a) mouse made by De Felipe et al. (1998) was used in experiments by Murtra et al. (2000) and others. This was bred with outbred MF1 mice to create the MF1 x 129/sv x C57BL/6 (MF1; b) mouse that was used by Ripley et al. (2002), Gadd et al. (2003), Gadd (2003) and Morcuende et al. (2003) as well as in this thesis. The original 129:B6 mouse was also backcrossed over 10 generations onto the C57BL/6 background to produce the B6 (c) mouse used in this thesis. Additionally, this mouse was then crossed back onto the 129/sv background over one generation to produce the C57BL/6 x 129/sv mouse (B6:129; d) which has also been used in this thesis.

2.1.3 *Breeding*

Mice were bred from homozygous breeding pairs. Homozygous mice from the F1 generation of heterozygous breeding pairs were frequently introduced into the colony to minimize the effects of genetic drift. Mice were weaned at approximately three weeks of age.

2.1.4 *Verification of genotype*

The genotype of all mice used was verified by PCR of tail-tip DNA. Three PCR primers were used: NK1-F, NK1-R and Neo-F. NK1-F and NK1-R amplify a 350-base section of exon 1 of the gene encoding the NK1 receptor gene. Neo-F and NK1-R amplify a 260-base section of the NK1 receptor gene including part of the inserted cassette. Resolution of these PCR products gives a single band of either 350 or 260 bases for homozygous $NK1^{+/+}$ or $NK1^{-/-}$ mice, respectively, whilst both bands are seen with the heterozygous $NK1^{+/-}$ mouse. Full genotyping method is given in section A.2 in Appendix A. Figure 2.3 shows an example gel on which bands corresponding to the different genotypes appear.

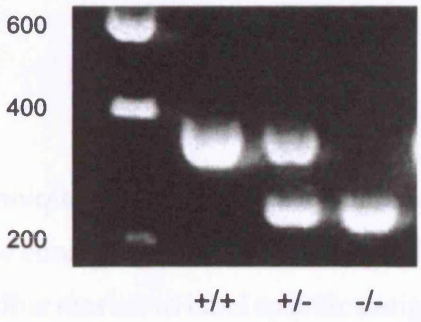

Figure 2.3: NK1 PCR for genotyping purposes. Two bands representing the PCR products of 350 and 260 bases can be seen alongside the molecular ladder. The band of 350 bases represents $NK1^{+/+}$, the band of 260 bases the $NK1^{-/-}$, and both bands the heterozygote.

2.1.5 *Culling*

Mice were killed either by decapitation, CO_2 asphyxiation or via perfusion. Mice killed by asphyxiation were placed in a chamber and the concentration of CO_2 was slowly raised. When the animals had stopped breathing, death was verified by physical breaking of the neck, exsanguination or removal of the brain.

2.1.6 *Perfusions*

Perfused mice were terminally anaesthetised by intraperitoneal (i.p.) injection of 0.2 ml pentobarbitone sodium (200 mg/ml; Euthatal; Rhone Merieux, Harlow, UK) before being perfused intracardially with 10 ml of heparinised PBS, followed by 100 ml ice-cold 4 % paraformaldehyde (PFA; BDH) solution in 0.1 M PB.

2.1.7 *Drugs*

Morphine sulphate was purchased from Sigma (Poole, UK). It was dissolved in sterile 0.9 % saline and administered i.p.. All injections were given in a volume of 6.67 ml/kg. Doses were calculated as the free-base.

2.2 *Molecular techniques*

2.2.1 *Immunocytochemistry*

Immunohistochemistry (IHC) was the main technique used for identification and quantification, in a region-specific manner, of tissue constituents in this thesis. In IHC, specific constituents can be identified by using a visible marker to label specific antigen-antibody binding. Specific antibodies to proteins are raised by injecting an animal such as a goat, a sheep or a rabbit with a short peptide corresponding to part of the sequence of the protein under investigation conjugated to a large carrier protein such as bovine serum albumin (BSA). Plasma is collected from the animal and antibodies

present are purified. Unlabelled primary antibodies are applied to tissue sections where they will bind the appropriate antigen. Often a second amplification step is added using the avidin/biotin system. This extra step amplifies the signal by exploiting the high affinity of the egg white glycoprotein avidin for the small molecular weight vitamin, biotin. Both avidin and biotin can be easily conjugated to other molecules and each avidin molecule is able to bind four biotin molecules. Biotinylated secondary antibodies, raised to antibodies from the primary antibody's host, can be used to link avidin-bound visible markers to the antigen of interest. These visible markers can either be chromogenic, radiographic or fluorescent, as described below. Additional amplification steps, such as tyramide signal amplification (TSA), may also be used to improve the signal-to-noise ratio.

2.2.1.1 Chromogenic staining (DAB)

3,3'-diaminobenzidine tetrahydrochloride (DAB) has been used as a label for most of the IHC in this thesis. It is a chromogenic electron donor in the presence of the enzyme horseradish peroxidase (HRP) which catalyses its oxidation to create an insoluble brown polymer. By using an HRP-avidin complex this reaction product can be used to visualise complexes surrounding the primary antibody. Nickel ions can be added to the reaction mixture to produce a more intense grey-black precipitate. DAB is highly carcinogenic so great care must be taken using and disposing of it.

2.2.1.2 Fluorescent staining

Fluorescent dyes bound to avidin are often used to visualise labelled antigens. As different dyes can be excited by different wavelength light they may also be used to label separate antigens in the same tissue. Photomicrographs can then be merged to inspect the degree of colocalisation of the antigens.

Table 2.1: Primary antibodies

Antigen	Source	Host animal	Detection method	Optimal dilution
NK1 receptor	Eurogentec	Rabbit	DAB, FITC	1:5000
Glucocorticoid receptor	Abcam	Rabbit	Alexa488	1:1000
Bromodeoxyuridine	Beckton Dickinson	Rat	DAB	1:5000

2.2.1.3 *Tissue preparation*

Animals were perfused as described in Section 2.1.6. Perfused tissue was dissected and post-fixed for between 2 h and 24 h in 4 % PFA. Brains were cryoprotected in 30 % sucrose in 0.1 M PB containing 0.02 % NaN_3, and stored at 4 °C for at least 12 h until being sectioned.

Brains were mounted onto a pre-cooled sledge microtome (SM 2000R; Leica Microsystems, Milton Keynes, UK) and frozen with dry ice. Sections were cut coronally at 40 μm, collected in 5 % sucrose in 0.1 M PB containing 0.02 % NaN_3, and stored at 4 °C.

Before use in immunocytochemistry, all primary antibodies were tested at a range of dilutions, using different conditions and detection methods to optimise their use.

A detailed method for all IHC experiments is given in Appendix A.

2.2.1.4 *Specificity of the glucocorticoid antibody*

As the antibody for the glucocorticoid receptor had not been used in our laboratory before, an immunoblot was conducted to ensure binding to an antigen of appropriate size and distribution was observed. A detailed protocol for the immunoblotting procedure is given in section A.4 in Appendix A.

Figure 2.4 shows an immunoblot conducted on various brain regions with a band corresponding to a protein of the same size as GR (97 kDa) and with appropriate distribution throughout the central nervous system (De Kloet and Reul, 1987).

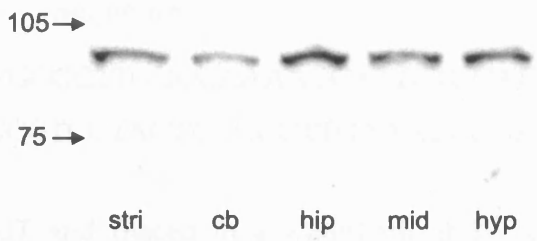

Figure 2.4: Immunoblot to test specificity of the GR antibody. A band is seen representing the 97 kDa protein in all brain areas examined. stri, striatum; cb, cerebellum; hip, hippocampus; mid, midbrain; hyp, hypothalamus.

2.2.2 In situ *hybridisation*

Autoradiographic *in situ* hybridisation was the main technique used for identification and quantification of RNA in this thesis. Radioisotope-labelled antisense oligonucleotides corresponding to a section of the gene sequence of interest are hybridised to tissue sections. Exposure to photographic film results in a regionally-specific and semi-quantifiable image being produced on the film.

2.2.2.1 *Tissue preparation*

Brains were removed after decapitation and frozen in isopentane maintained at −45 ±5 °C. Tissue was mounted onto the chuck of a cryostat (CM1900, Leica Microsystems) and allowed to warm to the temperature of the chamber (−22 °C). 15 μm coronal sections were cut and thaw-mounted onto poly-L-lysine coated slides (BDH). Sections were allowed to air-dry at ambient temperature for at least 1 h before being fixed in sterile 4 % PFA in 0.1 M PBS for 5 min, washed in sterile 0.1 M PBS for 1 min, and dehydrated in sterile 70 % ethanol for 5 min. Slides were transferred to sterile 95 % ethanol and stored at 4 °C until *in situ* hybridisation was performed.

2.2.2.2 *Probe labelling*

Antisense probes (table 2.2) were labelled using ^{35}SdATP (Perkin Elmer, Beaconsfield, UK) and terminal deoxytransferase (rTdT; Promega, Southampton, UK). Oligonu-

Table 2.2: Antisense probes

Molecule	Sequence
CRF	TCGGGAAATGAAATGTTGCGCTTGGCCAAAACGATTCTGCATTTA
cFos	GCAGCGGGAGGATGACGCCTCGTAGTCGGCGTTGAAACCCGAGAA

cleotide was added to isotope and rTdT and placed in a waterbath at 37 °C for 30 – 60 min before the reaction was quenched with 40 μl TE. The resulting solution was spun through a column (Micro Bio-Spin P-30 Tris, Bio-Rad, Hemel Hempstead, UK) to remove unbound isotope and the amount of labelling was quantified using a scintillation counter (LS60001C, Beckman Coulter, High Wycombe, UK). Probes had 1 μl dithiothreitol (DTT; Sigma) added to them and were stored at –20 °C until use.

Probe sequences were compared to other genomic sequences to ensure that they were unique. Pilot studies with the CRF probe showed that its expression was highly localised to the PVN. Pilot studies using the cFos probe showed that its expression was upregulated in the striatum 2 h after cocaine treatment compared with saline treated animals (data not shown). Additionally, no expression of cFos mRNA was seen in the PVN of unstressed animals.

Non-specific binding controls were also performed and showed no labelling of sections for either probe.

2.2.2.3 *Hybridisation*

Sections were removed from storage under ethanol and allowed to airdry for 1 – 2 h. A hybridisation mixture was prepared by adding the labelled probe and DTT to a hybridisation buffer. Non-specific binding (NSB) controls were also prepared by adding an excess of unlabelled oligonucleotide to the solution. In these slides the unlabelled oligonucleotide should outcompete the radiolabelled oligonucleotide. The appropriate mixture was applied to the slides which were then coverslipped and left in

a hybridisation oven at 42 °C overnight. The following day slides had their coverslips removed and were washed twice for 30 min in 1 x SSC buffer (National Diagnostics, Hull, UK) at 55 °C, once in 0.1 x SSC at room temperature, and dehydrated in 70 % ethanol and 95 % ethanol. Slides were then left to air-dry before being secured in a cassette. A piece of photographic film (Kodak BioMax MR; Sigma) was placed in the cassette for 1–2 weeks exposure.

2.2.2.4 *Emulsion-dipping*

To enable higher resolution of probe labelling including specific cellular distribution, sections were exposed to photographic emulsion and developed. This allowed sections to be examined under a microscope. In the dark, slides were dipped in pre-warmed emulsion (K5; Ilford, Mobberley, UK) and allowed to dry. Slides were kept in light-tight boxes at 4 °C for 5 times the length of time that they were exposed to film. Subsequently, slides were developed with Kodak D19 developer (Sigma) and counter-stained with thionin.

A detailed method for probe labelling, hybridisation and emulsion-dipping is given in section A.5 in Appendix A. Analysis of autoradiographic films is detailed in section 2.2.3.

2.2.3 *Microscopy and Photography*

Antibody-labelled sections were visualised using a Leica DMR microscope (Leica Microsystems) or a Nikon Eclipse E800 microscope (Nikon, Kingston-upon-Thames, UK). For chromogenically-labelled antigens bright-field conditions were used, whereas fluorescent dyes were visualised under the appropriate excitation from a 50 W mercury lamp (HB050; Osram, Munich, Germany). Bright-field photography was carried out using a Photometrics Coolsnap CF camera attached to a Dell PC running MicroComputer Imaging Device Basic 7.0 (MCID Basic 7.0; Imaging Research, Amersham, UK).

Images on photographic film were analysed using a Northern Light B90 light box

(Imaging Research) and a MicroNikkor 55 mm CCD video camera (Nikon). Analysis of optical density was carried out using MCID Basic 7.0 (Imaging Research). Areas of interest were quantified using densitometric analysis.

To measure luminosity of cells a confocal microscope was used (Bio-Rad 600, Bio-Rad). Cells were visualised at x 60. Background measurements were subtracted from the mean cell luminosity on each separate section.

2.3 *Corticosterone measurements*

For all experiments examining the HPA axis detailed in Chapter 5, animals were handled for 5 d prior to blood sampling or tissue collection to minimize handling-evoked stress responses. Handling consisted of removal from the home cage and transport to and from the procedure room before being returned to the home cage. This was performed by the same experimenter on all days.

On day 6 control animals were removed from the home cage, taken to the procedure room and rapidly killed by cervical dislocation. Animals undergoing restraint were taken to a different room and restrained in a 50 ml Falcon tube with an airhole for 30 min prior to being killed by cervical dislocation or being removed and placed back in a cage for 30 – 60 min.

Blood was taken via cardiac puncture, collected into EDTA-coated microvette tubes (Sarstedt, Numbrecht, Germany) and placed on ice until all samples were collected. Tubes were then spun at 13,000 g for 15 min to separate plasma. The plasma was stored at –20 °C prior to analysis for corticosterone levels.

Corticosterone was measured via radioimmunoassay. Plasma was added to an acidic citrate buffer. The acidity of the buffer causes all bound corticosterone in the whole blood samples to dissociate from capillary blood glucose (CBG). These samples were then loaded in triplicate with the radioactive tracer (^{125}I-corticosterone) and specific antibody (courtesy of Dr Gabor Makara, Institute of Experimental Medicine, Budapest Hungary). Samples were covered and incubated at 4 °C overnight. A

standard curve was also prepared. The following day activated charcoal solution was added to all tubes. Charcoal-filled samples were then vortexed and centrifuged at 4,000 rpm for 15 min at 4 °C. The supernatant containing all antibody-bound corticosterone was removed and discarded via aspiration leaving all "free" corticosterone bound to the charcoal pellet. The level of radioactivity in each sample was then measured using a gamma counter.

An 11 sample standard curve was set up using serially diluted corticosterone standards (Sigma), ranging from 10 ng/ml to 0.0970 ng/ml. These were set up in parallel to the samples except that they were assayed in triplicate. Also counted were three tubes containing pure tracer and three tubes containing tracer and buffer. Quality control samples (from CORT stock; Sigma) were also counted.

2.4 *Behavioural Techniques*

Behavioural experiments using animals are useful for studying how underlying cellular processes affect the whole organism and interact at the systems level. However, the complexity of animal behaviour means that there are many confounding factors that need to be taken into account when conducting behavioural work. Of these the animal's relation to its environment is probably the most important. This includes the nature of the environment as well as how the individual animal reacts to it. As such stress and anxiety about features in the environment can drastically affect an animal's behaviour in an unpredictable way. Because of this it is important to keep these features well-controlled so that they do not become confounds. A few examples are given here.

2.4.0.1 Habituation

Habituation to the experimental room and/or equipment is vital (Leussis and Bolivar, 2006). Animals find novel environments very stressful and unless the object is to study an animals' behaviour under these conditions a habituation period is generally necessary. Habituation to handling and experimental procedures such as injections

may also be required to prevent confusing effects of stress and anxiety with results of a treatment.

2.4.0.2 *Timings*

The time of day and day of the week are both very important for animal experiments. Circadian changes in steroid levels affect behaviour over the course of the day and routine patterns of activity in the housing facilities such as cleaning out or feeding will also disturb animals.

2.4.0.3 *Sensory cues*

Species differ in how they use and relate to their senses. For example, compared to humans, rodents have a highly developed sense of smell. Olfaction forms a large component of their sensory experience and they are highly sensitive to odours in their environment. Changes that are undetectable to the experimenter may provide the animal with a different set of cues that may impact on the experiment. Keeping disturbances to other sensory modalities to a minimum will also minimize animals' anxiety levels, for example, keeping noise levels constant.

All behavioural experiments were carried out in the same room under fluorescent strip lighting at an intensity of 40 lx except where indicated

2.4.1 *EthoVision*

To monitor animals' behaviour a computerised tracking system was used for many experiments (EthoVision 2.3; Noldus Information Technology, Waginen, The Netherlands). A black and white CCD camera (High Resolution B/W CCD Camera Model VCB-3372P; Sanyo Electric Company, Osaka, Japan) attached to a computer was used to track the animal's position by detecting differences from a standardised background

image. The camera converted the signal from analogue to digital and sampling was conducted at a frequency of 5 Hz. The digitised image was subtracted from a previously collected reference image to determine the position of the animal within the arena. The construction of the arenas and camera set-up allowed the simultaneous recording of up to 8 animals. For some experiments an infrared lightbox was used to illuminate the arenas from below. By attaching an infrared filter to the camera a 'shadow' of the animals against the background was produced. This prevented problems due to reflections from the lighting in the room and difficulties in detecting animals with a coat colour that matched the background. It also enabled filming of animals under lower light intensities. A variety of parameters could be subsequently analysed such as average speed, distance to a point or time spent in a particular zone. Figure 2.5 shows an example screen shot from an experiment using EthoVision.

2.4.2 Drug-induced locomotion

Most drugs of abuse at certain doses will produce hyperlocomotion when administered to animals. This is often due to the sustained release of dopamine in the striatum and so may be related to the same neural substrates that mediate the rewarding or addictive properties of a drug. The extent of this locomotor activation can therefore be used to elucidate certain properties of the drug or effects of a particular manipulation on these properties. See section 3.1.1 in the following chapter for more details.

Locomotor responses elicited by morphine were assessed in black Perspex boxes (internal dimensions: 9 cm [w] x 20 cm [l] x 11 cm [h]). After at least 1 h habituation to the experimental room, animals were placed in the arenas and activity was monitored for 1 h using EthoVision. After this, animals were removed, injected with drug and placed back in the same arena. Recording via EthoVision resumed immediately for a further 2 h. A video camera attached to a computer running EthoVision 2.3 (Noldus Information Technology) was used to monitor the position of the mice and locomotor activity was assessed by calculating the mouse's mean speed in mm/s over the 15 min

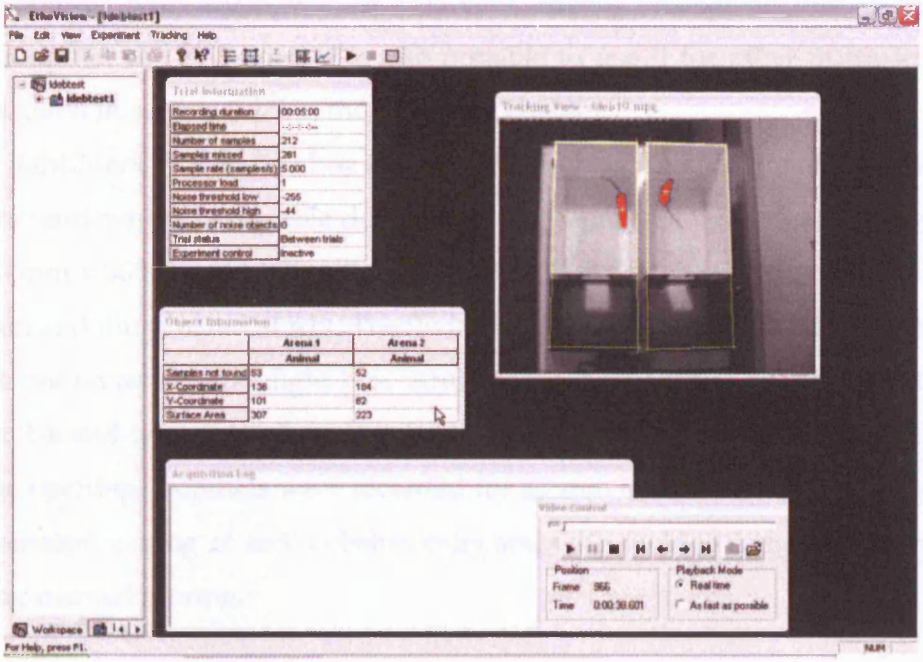

Figure 2.5: A typical screenshot of EthoVision recording a LDEB experiment. Animals were detected via subtraction from the background image and are seen here highlighted in red. Various parameters could subsequently be analysed, for example, distance moved, time in zones or velocity.

recording session. All the activity tests were carried out between 10.30 a.m. and 13.30 p.m.

2.4.3 *Light/dark exploration box*

The light/dark exploration box (LDEB) is a simple piece of equipment consisting of two differently-lit compartments with a shuttle door joining them. Under a certain set of parameters, depending on the experiment, animals will be encouraged to explore the two chambers for a set period of time. The test exploits a conflict between the rodent's innate aversion to bright lights and their innate tendency to explore a

novel environment (Bourin and Hascoet, 2003). It has mostly been used as a screen for compounds with anxiogenic or anxiolytic effects. However, due to the many behaviours that can be scored it is also possible to use it for other purposes. More detail is given in section 3.1.2 in the following chapter.

The light/dark exploration box (LDEB) was made of Perspex and divided into two compartments with a removable door separating them (see figure 2.6). The light side was 200 mm x 300 mm and illuminated at 20 lx. The dark compartment was 150 mm x 200 mm and illuminated at 5 lx. The floor of each box was transparent and the boxes were placed on an infrared light box. Animals were allowed to habituate to the dark zone for 1 h and were then placed in the centre of the light compartment facing away from the opening. Animals were recorded for 30 min both with EthoVision software for automated scoring of certain behaviours and with a video camera for behaviours requiring manual scoring.

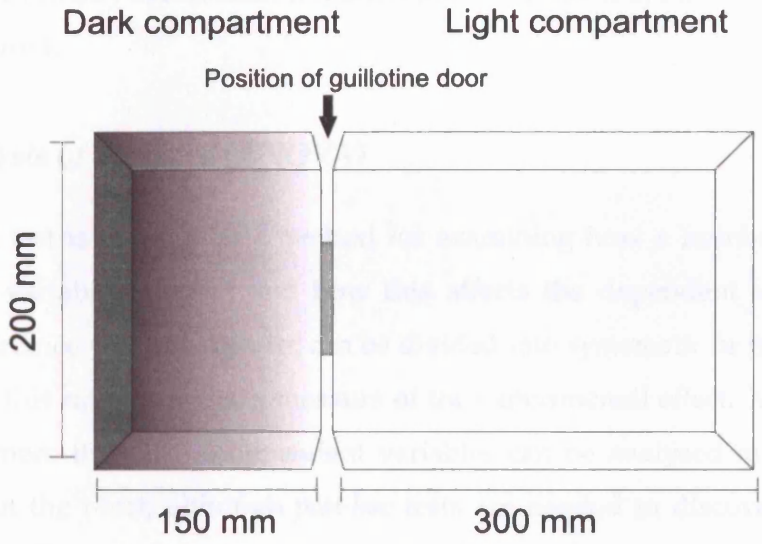

Figure 2.6: The light/dark exploration box

2.5 Statistical analysis

A number of different statistical tests were used to analyse the data presented in this thesis. In this section the general principles underlying these tests are described and some general rules that were used throughout. Specific tests are described in more detail in their relevant chapters.

2.5.1 Students t-test

The t-test is a method of comparing the means of two data sets. If the data sets arise from the same population then their means are unlikely to differ greatly, except by chance. The standard error is used to gauge the natural variability within the sample set so that the extent of variability expected in the means can be calculated. T-tests can be either paired or unpaired depending on whether the two samples are related or independent. The t-test is a parametric test meaning that it is assumed that the population is normally distributed. If this assumption is not met then a non-parametric test must be used.

2.5.2 Analysis of Variance (ANOVA)

The ANOVA test is a parametric method for examining how a number of different independent variables interact and how this affects the dependent variable. The amount of variance within a data set can be divided into systematic or non-systematic variance and this ratio provides a measure of the experimental effect. More than two groups and more than two independent variables can be analysed making it more powerful than the t-test, although *post-hoc* tests are needed to discover the specific relationships between different groups. Repeated measures ANOVA tests can be used to analyse how the same subjects respond under a number of different conditions, for example, in a time course study. Analysis of covariance (ANCOVA) tests can be used to remove the effect of a particular experimental variable from the overall effect on the

dependent variable. Multivariate ANOVA (MANOVA) tests can be used to prevent false positives resulting from the analysis of several dependent variables within the same data set.

All statistical analysis in this thesis was carried out using SPSS 12.0.1 for Windows (SPSS Inc., Chicago, US). The data were checked for violation of the assumptions of the test procedures such as homogeneity of variance and Normality of the error. Where these assumptions were not upheld, the data were transformed using square root, logarithmic, or inverse transformations as appropriate or an alternative statistical test was used. $P<0.05$ was the criterion for statistical significance.

Chapter 3

Results I: Behavioural differences in MF1 and B6 mice

3.1 *Introduction*

This chapter describes a set of experiments comparing the behavioural phenotype of the $NK1^{-/-}$ mouse on two different genetic backgrounds. The original mouse created by De Felipe et al (1998) was on a mixed 129:B6 background. To partially eliminate the influence of flanking genes on the phenotype, this mouse was crossed with the outbred MF1 strain to give a mixed MF1:129:B6 (MF1) mouse. Work using the resulting mouse corresponded closely with previous studies (De Felipe et al., 1998; Murtra et al., 2000; Ripley et al., 2002; Gadd et al., 2003; Gadd, 2003). However, this was a suboptimal solution to the problem caused by flanking genes. Additionally, it performed poorly on cognitive tests such as the Morris water maze. C57BL/6 mice are known to outperform other inbred strains on these tasks. Therefore, to reach genetic homogeneity of background and to enable investigation into the cognitive effects of NK1 receptor knockout a backcrossing strategy was adopted in parallel. This involved backcrossing over 10 generations onto the C57BL/6 background (B6).

The experiments described below compare the effect of NK1 receptor knockout in the mixed MF1 hybrid and the B6 strain. Morphine-induced hyperlocomotion and behaviour in the light/dark exploration box (LDEB), a test commonly used to screen for anxiety-like behaviours, are examined.

3.1.1 *Morphine-induced hyperlocomotion*

As with many drugs of abuse, administration of morphine at certain doses produces a hyperlocomotor response in animals. This is thought to be related to the rewarding properties of the drug due to similarities in the underlying neural substrates (Robinson and Berridge, 2003). The hyperlocomotion is thought to result from increased activation of the mesolimbic dopaminergic system which projects ventrally from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc), the caudate putamen, and the prefrontal cortex (Wise, 1998). Morphine activates this system by inhibiting GABAergic interneurons in the VTA by binding to μ-opioid receptors. This results in a disinhibition of the dopaminergic projection cells and elevated dopamine (DA) release in the NAcc (Johnson and North, 1992). It is this system that is believed to become sensitised in drug addiction when the resulting homeostatic plasticity manifests as pathology (Berke and Hyman, 2000). However, other factors that are specific to the species and strain of the animal, or to the testing conditions and protocol will be involved in the expression of this hyperlocomotion. As such, morphine also acts at numerous other sites within the brain and may modulate other aspects of an animal's behaviour such as anxiety (Costall et al., 1989; Dockstader and van der Kooy, 2001). Furthermore, a study by Murphy et al. (2001) that tested three inbred strains showed that although morphine increased striatal dopamine in all of them, there was no correlation with the locomotor response. This implies that locomotion is subserved by a number of different systems and is likely to be modulated in a complex manner. Additionally, other sites of action may important in mediating the rewarding properties of morphine. Although lesions of the NAcc profoundly disrupt conditioning to psychostimulant drugs, morphine-induced place preference has been shown to be unaffected, or at most attenuated (Olmstead and Franklin, 1996).

As discussed in the previous chapter the $NK1^{-/-}$ mouse has been shown to have a reduction in the rewarding, reinforcing and locomotor responses to opiates (Murtra et al., 2000; Ripley et al., 2002; Gadd, 2003). Additionally, rats given NK1 receptor

antagonists have an attenuated morphine-induced locomotor response and an increase in self-administration of heroin (Placenza et al., 2006). We therefore decided to compare morphine-induced hyperlocomotion between the genotypes in the MF1 and B6 strains.

3.1.2 The light/dark exploration box (LDEB)

The LDEB is a test that has most commonly been used to screen for compounds with anxiolytic or anxiogenic properties. Rodents have an innate aversion to brightly-lit open spaces as well as a tendency to explore novel environments in response to mild stressors (Crawley and Goodwin, 1980). A conflict between the tendency to explore and the tendency to avoid the unfamiliar (neophobia) is set up and the resulting exploratory activity reflects the combination of these two tendencies (Bourin and Hascoet, 2003). The standard test consists of a brightly lit chamber connected to a smaller darkened one by a door. Behaviours in each chamber can be measured. Modifications to the apparatus can include changes in the sizes and illumination of the compartments (Costall et al., 1989; Young and Johnson, 1991) and the presence of a tunnel joining them (Belzung et al., 1987). Traditionally, the number of transitions between the two compartments, independent of increases in locomotion, was used as an index of increased exploratory behaviour. Increases in exploratory behaviour have been linked to an anxiolytic profile in many tests (Crawley and Goodwin, 1980). Other researchers have used different spatiotemporal parameters as indices for anxiolysis-induced exploration including, time spent in each compartment and latencies to leave or return to each compartment (Costall et al., 1989; Bourin and Hascoet, 2003). Ideally, many more active and passive behaviours can be scored in parallel and provide a more sophisticated profile for the animal (Rodgers, 1997).

Many drugs have been tested using the LDEB. Anxiolytic drugs, especially the benzodiazepines, cause the most reliable changes in behaviour and will generally increase exploratory behaviours (Bourin and Hascoet, 2003). Drugs working at most of the different 5-HT receptors have also been extensively studied using this paradigm.

Agonists at the 5-HT_{1A} and 5-HT_{1B} receptors have the most consistent anxiolytic profiles (Costall et al., 1989; Metzenauer et al., 1992; Hascoet and Bourin, 1998) whilst antagonists at the 5-HT_3 receptor have also been demonstrated to have anxiolytic effects (Costall et al., 1989; Bill et al., 1992).

Other factors related to the strain and age of the mice being used are also known to impact on results in the LDEB (Crawley et al., 1997; Hascoet and Bourin, 1998; Hascoet et al., 1999; Bourin and Hascoet, 2003). These may often interact with each other or other manipulations such as a targeted genetic mutation or anatomical lesion. Large differences have also been noted between laboratories and experimenters (Wahlsten et al., 2003). Taken together all these features mean that comparison between different studies is often difficult, if not impossible. Assertions that any one behaviour, such as transitions, can be taken as a strict representation of anxiety are dangerous. To prove an anxiolytic effect therefore the best that can be done is to use a proven anxiolytic such as diazepam as a positive control. Even this situation is suspect as it relies on the telelogical assumption that because diazepam is known to reduce anxiety in humans it will reduce anxiety in mice. Using these assumptions only drugs with the same mechanism of action as ones currently available may be picked up and so give false negatives in some cases (Belzung and Griebel, 2001).

Another important consideration when using the LDEB is that locomotor behaviour can often be a confounding factor (Bourin and Hascoet, 2003). Drugs or manipulations that alter spontaneous locomotion will cause changes in other behaviours that may be associated with other affective states. It is important to remove the effect of locomotion either by using it as a covariate when analysing or by prior screening for locomotor effects.

The LDEB has been used in our laboratory as an ethological behavioural screen that does not require prior training of the animals. Previously, differences in behaviour between $NK1^{-/-}$ and wildtype mice have been found in the LDEB. In one study, the $NK1^{-/-}$ mouse on the MF1 background was found to have a reduction in risk assessment behaviours and an increase in locomotor activity. Removal of locomotor

activity as a covariate revealed that transitions and rearing also differed between the genotypes and were previously masked by the increases in locomotion (Herpfer et al., 2005). More recently, similar results were found for risk-assessment behaviours and locomotor activity although no difference was found in rearing once locomotor activity had been removed. Additionally, $NK1^{-/-}$ mice were found to spend less time in the light zone (Fisher, 2005). Both studies implicated differences in noradrenergic function in the knockout mouse through the use of desipramine (Herpfer et al., 2005) and the α-2 adrenoceptor antagonist RX821002 (Fisher, 2005).

3.2 Materials & Methods

3.2.1 Subjects

Mixed 129:B6 hybrid $NK1^{-/-}$ mice crossed onto the MF1 background for 1 generation (MF1) or onto the C57BL/6 background for 10 generations (B6) were compared here. More detail is given in section 2.1.2.

3.2.2 Morphine-induced locomotion

EthoVision was used to monitor animals behaviour. Animals were allowed to habituate to the activity boxes for 1 h before being injected with saline or morphine and recorded for 2 h. Figure 3.1 shows this protocol. Locomotor activity was recorded continuously and binned into 15 min periods for plotting and analysis. Each strain was analysed separately using 3-way repeated measures ANOVA with genotype and dose as the between subjects factors and time as the within subject repeated measure. Significant main effects or interactions were then investigated further using 1-way ANOVA on individual time points with group as the between subjects factor and *post hoc* Tukey tests. Dose response data for B6 mice were analysed using 2-way ANOVA with genotype and dose as the between subjects factors and *post hoc* Dunnett's tests. Additionally, data from the habituation period was collapsed over all dose groups and

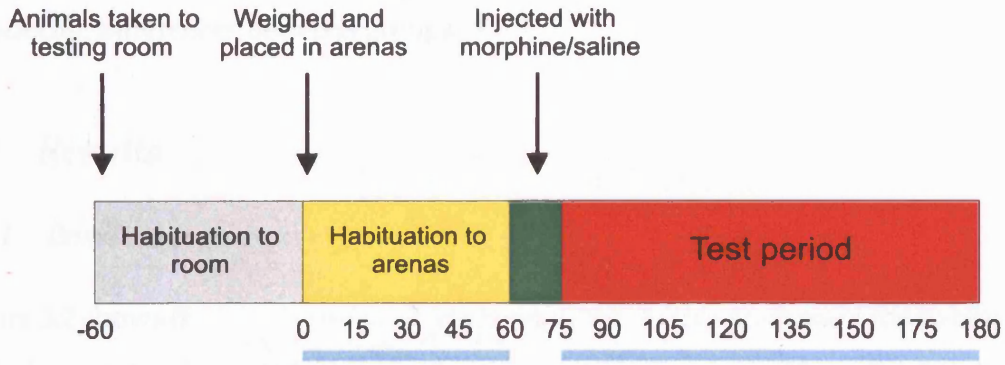

Figure 3.1: Protocol of morphine-induced locomotion experiments. Animals were taken to the testing room 1 h before the start of the experiment (grey box). Animals were weighed, placed in arenas and activity was monitored for 1 h (yellow box). Animals were removed from boxes and injected with saline or morphine (green box). Animals were placed back into the same arena and activity was recorded for a further 2 h (red box). Timings in minutes are shown at the bottom of the diagram. The cyan bar underneath represents the time during which animals were recorded.

1-way ANOVA with genotype as the between subjects factor was used to investigate changes in baseline locomotion in each strain.

3.2.3 The LDEB

Animals were allowed to habituate to the dark compartment with the door closed for 1 h before being placed in the centre of the light zone and having their behaviour recorded for 10 min. Locomotor activity and time spent in each compartment was measured automatically using EthoVision while all other behaviours were scored by a experimenter blinded to treatment. A MANOVA test was initially used to analyse all behaviours and reduce Type I errors. Significant behaviours were subsequently analysed using 2-way ANOVAs with genotype and strain as between subject factors and using 2-way ANCOVA with locomotor activity as a covariate. 1-way ANOVA with group as the between subjects factor and *Post hoc* Tukey tests were used to look

for specific differences between groups.

3.3 Results

3.3.1 Baseline locomotion

Figure 3.2 shows the baseline locomotion in each strain from analysis of the habituation period and after saline injection. In the B6 strain, 1-way ANOVA on the habituation period revealed no main effect of genotype ($F_{1,60}$=3.746, p=0.058). In contrast, in the MF1 strain, 1-way ANOVA revealed that NK1$^{-/-}$ mice had a higher baseline locomotion than wildtype counterparts during the habituation period ($F_{1,22}$=12.000, p=0.002). Analysis of the test period of animals administered saline also showed the same pattern. 1-way ANOVA revealed an effect of genotype in the MF1 mice ($F_{1,10}$=8.300, p=0.016) but not in the B6 strain ($F_{1,16}$=2.340, p=0.146). Under these test conditions NK1$^{-/-}$ mice in the MF1 strain displayed higher locomotor behaviour than wildtype MF1 mice.

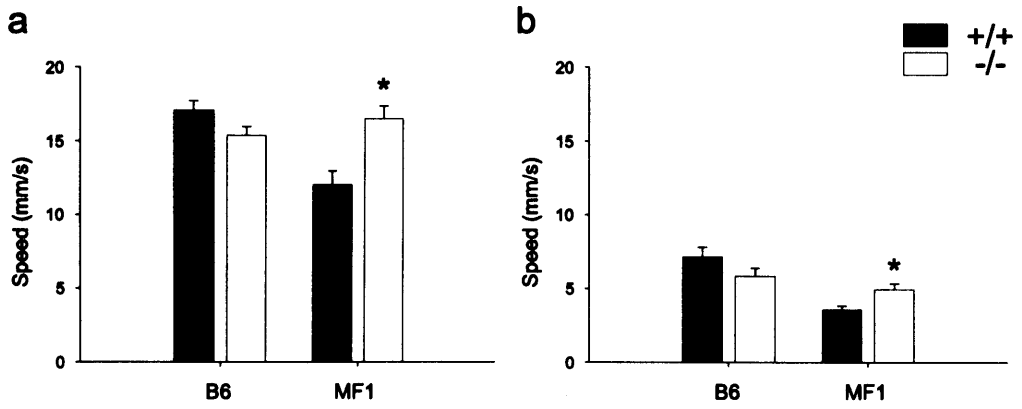

Figure 3.2: Baseline locomotion. Mean ± SEM speed travelled by NK1$^{-/-}$ and wildtype mice on B6 and MF1 backgrounds during the habituation period (a) and after administration of saline (b) *P<0.05 vs. +/+; n=6-25.

3.3.2 *Morphine-induced locomotion*

Figure 3.3 shows the results of the morphine-induced hyperlocomotion experiment in the $NK1^{-/-}$ and wildtype mice on the MF1 and B6 backgrounds.

3.3.2.1 *B6 mice*

Three-way repeated measures ANOVA with genotype (G) and dose (D; 0 vs. 20 mg/kg) as between subjects factors and time (T) as the within subjects factor revealed significant main effects of time and dose and a significant T x D interaction. These data are summarised in table 3.1.

Subsequent 1-way ANOVA tests on each time point with group (wildtype morphine vs. wildtype saline vs. $NK1^{-/-}$ morphine vs. $NK1^{-/-}$ saline) as the between subjects factor were performed and revealed that dose had a significant effect at all time points except for 15 min. *Post hoc* Tukey tests revealed that morphine-treated animals moved faster than saline-treated regardless of genotype.

Table 3.1: Results of 3-way ANOVA of morphine-induced locomotor data in B6 mice

Factor	d.f.	Error d.f.	F	P
Genotype (G)	1.000	26.000	1.090	0.306
Dose (D)	1.000	26.000	81.159	<0.001
Time point (T)	3.054	79.396	7.128	<0.001
G x D	1.000	26.000	0.004	0.949
G x T	3.054	79.396	0.545	0.656
D x T	3.054	79.396	83.614	<0.001
G x D x T	3.054	79.396	0.501	0.686

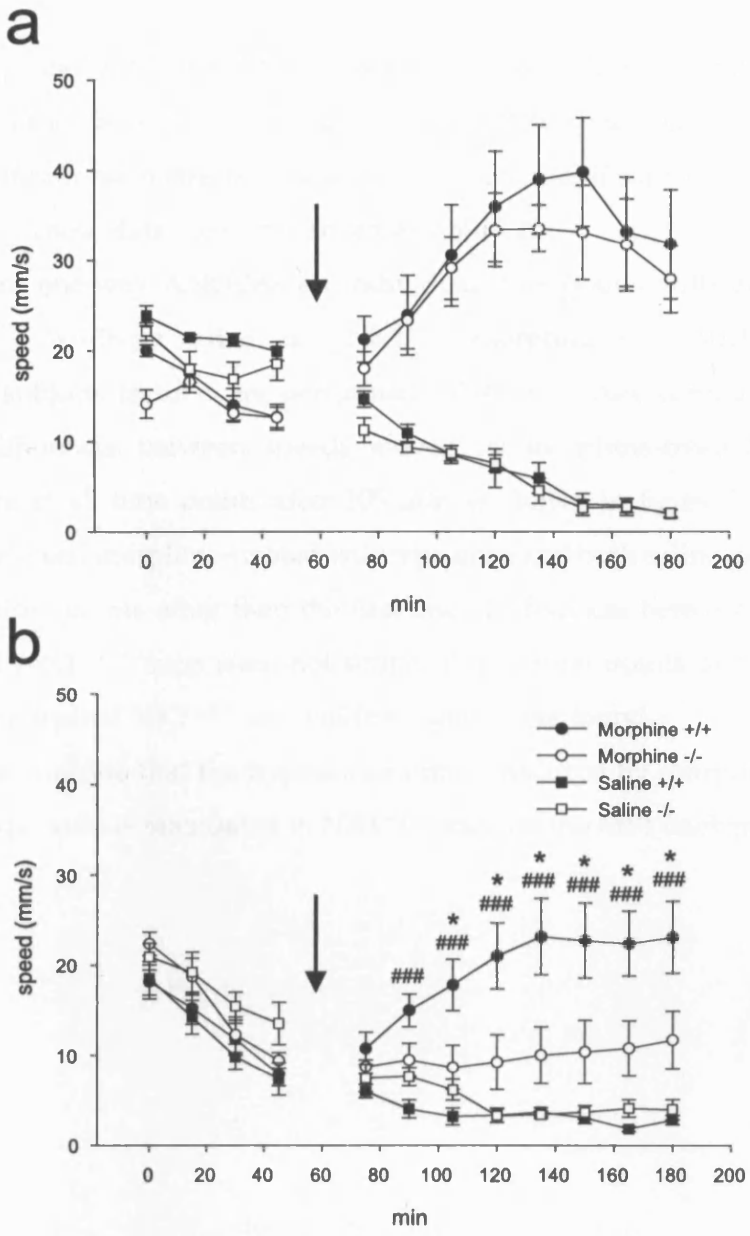

Figure 3.3: Morphine-induced locomotion. Mean ± SEM speed traveled by NK1$^{-/-}$ and wildtype mice on B6 (a) and MF1 (b) genetic backgrounds after administration of morphine (20 mg/kg) or saline. Arrows indicate time of injection. *P<0.05 vs. NK1$^{-/-}$; ###P<0.001 vs. saline; n=6-8.

3.3.2.2 *MF1 mice*

Three-way repeated measures ANOVA with genotype (G), dose (D; 0 vs. 20 mg/kg) as between subjects factors and time (T) as the within subjects factor on all time points revealed significant main effects of dose and time and significant T x G, T x D and G x D interactions. These data are summarised in table 3.2.

Subsequent one-way ANOVAs on individual time points with group (wildtype morphine vs. wildtype saline vs. $NK1^{-/-}$ morphine vs. $NK1^{-/-}$ saline) as the between subjects factor were performed. *Post hoc* Tukey comparisons revealed significant differences between speeds moved by morphine-treated $NK1^{-/-}$ and wildtype mice at all time points after 105 min as shown in figure 3.3b. Significant differences between morphine-treated wildtype mice and both saline groups were also found in all time points other than the first one. Differences between morphine and saline-treated $NK1^{-/-}$ mice were not found at any time points and no differences between saline-treated $NK1^{-/-}$ and wildtype mice were found.

These data indicate that the hyperlocomotion produced by morphine administration in wildtype mice is attenuated in $NK1^{-/-}$ mice on the MF1 background.

Table 3.2: Results of 3-way ANOVA on morphine-induced locomotor data in MF1 mice

Factor	d.f.	Error d.f.	F	P
Genotype (G)	1.000	20.000	1.121	0.302
Dose (D)	1.000	20.000	23.131	<0.001
Time point (T)	2.877	57.550	19.409	<0.001
G x D	1.000	20.000	8.232	0.009
G x T	2.877	57.550	6.788	0.001
D x T	2.877	57.550	14.792	<0.001
G x D x T	2.877	57.550	2.685	0.057

3.3.3 Dose response to morphine in B6 mice

No difference between the genotypes was seen in the hyperlocomotion induced by 20 mg/kg of morphine in the B6 strain. To test the hypothesis that this was a ceiling effect a number of different doses of morphine were tested.

2-way ANOVA with genotype (G) and dose (D) as between subjects factors revealed a main effect of dose but not genotype and no G x T interaction. These results are summarised in table 3.3. *Post hoc* Dunnett's tests showed that morphine increased locomotor activity at all doses compared with saline controls (5 mg/kg, $p=0.003$; 10 mg/kg, $p<0.001$; 20 mg/kg, $p<0.001$; 30 mg/kg, $p<0.001$). These data show that in the B6 strain morphine increases activity in wildtype and $NK1^{-/-}$ mice to the same extent.

Table 3.3: Results of 2-way ANOVA on morphine dose response in B6 mice

Factor	d.f.	Error d.f.	F	P
Genotype (G)	1	66	0.941	0.336
Dose (D)	4	66	57.485	<0.001
G x D	4	66	0.392	0.814

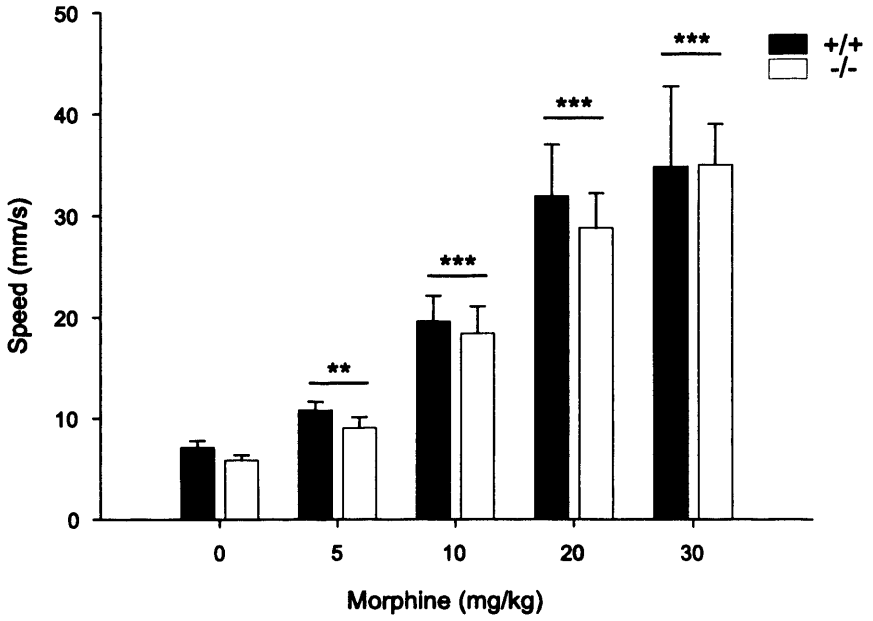

Figure 3.4: Dose response data for B6 mice after morphine administration. Mean ± SEM speed travelled in 2 h test period following injection. **, $p<0.01$; ***, $p<0.001$ vs. saline using Dunnett's *post hoc* test. N=5-9.

3.3.4 The LDEB

The LDEB was used to screen a number of behaviours to look for differences between the strains and genotypes. Table 3.4 gives the mean ± SEM data for all behaviours scored. These were first analysed with MANOVA to prevent generating false positives due to multiple comparisons. Significant behaviours were subsequently tested using two-way ANOVA with strain (S) and genotype (G) as the between subjects factors. These data are shown in table 3.5.

All the behaviours analysed subsequent to the MANOVA showed a main effect of strain (total activity, activity in the dark, number of transitions, stretch-attend postures (SAPs), total rears, rears in the dark, rears in the light, grooming bouts). Three behaviours showed a main effect of genotype (SAPs, rears in the dark and grooming

bouts). Four behaviours showed a G x S interaction (transitions, SAPs, total rears and rears in the light).

Because locomotor activity showed a significant effect of strain, further ANCOVA tests using locomotor activity as a covariate were performed. These data are given in table 3.6. After this procedure a main effect of genotype was revealed on total number of rears and the main effect of genotype on grooming bouts was lost. All the other behaviours remained as before. These results show that, with the exception of two behaviours, strain and genotype both have striking effects on a number of behaviours in the LDEB that are independent of the strain difference in locomotor activity.

Post hoc Tukey tests were conducted to investigate specific differences between the groups. The resulting data are summarised in table 3.5 and the significant results are shown graphically in figure 3.5 and described below.

Post hoc tests on the total activity revealed that, despite there being a main effect of strain, there were no significant differences between any of the groups.

Post hoc tests on the number of transitions, which showed a main effect of strain and a G x S interaction, revealed that there was no difference between genotypes on the MF1 strain but that $NK1^{-/-}$ mice on the B6 background performed significantly more transitions than their wildtype counterparts ($p=0.002$). Wildtype B6 mice also performed more than both MF1 genotypes (+/+, $p<0.001$; −/−, $p<0.001$) and $NK1^{-/-}$ B6 mice also performed more than wildtype MF1 mice ($p=0.011$).

The number of stretch-attend postures (SAPs), a form of risk assessment behaviour, showed a main effect of genotype, a main effect of strain and a G x S interaction. *Post hoc* tests revealed that wildtype MF1 mice performed significantly more SAPs than knockout MF1 mice ($p<0.001$) or either genotype on the B6 background (+/+, $p<0.001$; −/−, $p<0.001$). There was no difference between the B6 genotypes.

Post hoc tests on the total number of rears, which showed a main effect of strain and a G x S interaction, revealed that $NK1^{-/-}$ mice on the MF1 background performed significantly more rears than their wildtype counterparts ($p=0.020$) but that there was no difference between the genotypes on the B6 background. Both genotypes on the

Table 3.4: LDEB results in the MF1 and B6 strains. Means ± SEM are shown for each behaviour. Symbols indicate behaviours that showed a main effect of genotype (*), strain (†) or a significant interaction (‡) after analysis by ANOVA. The results of these analyses are shown in tables 3.5 and 3.6.

Behaviour	MF1 +/+		MF1 -/-		B6 +/+		B6 -/-	
Latency (s)	53.0	(20.5)	48.3	(13.1)	10.2	(1.8)	32.7	(15.1)
Return to light	16.2	(5.0)	12.7	(3.7)	9.4	(4.5)	11.6	(3.7)
Activity (cm/s) †	4.8	(0.5)	4.8	(0.2)	6.1	(0.2)	5.5	(0.2)
Activity (Dark) (cm/s) †	4.1	(0.6)	4.0	(0.3)	5.6	(0.2)	5.1	(0.2)
Activity (Light) (cm/s)	5.3	(0.6)	5.4	(0.2)	6.5	(0.3)	5.7	(0.2)
Time (Dark) (s)	181.6	(26.6)	177.9	(22.7)	191.7	(15.8)	170.3	(8.3)
Time (Light) (s)	404.1	(28.5)	410.7	(23.7)	387.8	(12.7)	423.8	(9.4)
Transitions †‡	22.0	(1.8)	31.8	(2.2)	58.3	(5.5)	38.5	(3.1)
SAPs * †‡	52.8	(7.6)	16.1	(2.6)	5.5	(1.5)	7.4	(2.4)
Rears (Total) †‡	29.5	(4.5)	46.5	(2.7)	62.4	(4.9)	59.9	(2.6)
Rears (Dark) * †	5.5	(0.8)	10.4	(1.7)	12.6	(1.6)	17.3	(2.0)
Rears (Light) †‡	24.0	(4.4)	36.1	(2.5)	49.8	(4.7)	42.6	(2.0)
Grooming (Bouts) * †	0.4	(0.2)	1.5	(0.8)	1.1	(0.4)	3.4	(1.0)
Grooming (s)	3.1	(2.3)	21.0	(12.8)	16.8	(8.3)	36.5	(13.3)

B6 background performed more rears than wildtype MF1 mice (+/+, $p<0.001$; −/− , $p<0.001$) and wildtype B6 mice performed more than $NK1^{-/-}$ mice on the MF1 background ($p=0.032$).

Post hoc tests on the number of rears in the dark, which showed a main effect of strain and of genotype, revealed no differences between the genotypes of each strain. $NK1^{-/-}$ mice on the B6 background performed more rears in the dark than either genotype on the MF1 background (+/+, $p<0.001$; −/−, $p=0.023$) and wildtype B6 mice performed more than wildtype MF1 mice ($p=0.018$).

Table 3.5: 2-way ANOVA on behaviours in the LDEB

Behaviour	Genotype		Strain		Interaction	
Total activity	$F_{1,28}$=0.663	P=0.422	$F_{1,28}$=7.938	P=0.009	$F_{1,28}$=0.992	P=0.328
Activity (Dark)	$F_{1,28}$=0.868	P=0.359	$F_{1,28}$=9.477	P=0.005	$F_{1,28}$=0.421	P=0.522
Transitions	$F_{1,28}$=2.106	P=0.158	$F_{1,28}$=38.941	P<0.001	$F_{1,28}$=18.328	P<0.001
SAPs	$F_{1,28}$=12.741	P=0.001	$F_{1,28}$=58.324	P<0.001	$F_{1,28}$=17.964	P<0.001
Rears (Total)	$F_{1,28}$=3.574	P=0.069	$F_{1,28}$=36.357	P<0.001	$F_{1,28}$=6.463	P=0.017
Rears (Dark)	$F_{1,28}$=9.005	P=0.006	$F_{1,28}$=19.556	P<0.001	$F_{1,28}$=0.006	P=0.938
Rears (Light)	$F_{1,28}$=0.486	P=0.492	$F_{1,28}$=20.208	P<0.001	$F_{1,28}$=7.200	P=0.012
Grooming (Bouts)	$F_{1,28}$=4.867	P=0.036	$F_{1,28}$=6.050	P=0.020	$F_{1,28}$=0.303	P=0.587

Table 3.6: 2-way ANCOVA using locomotor activity as a covariate on behaviour in the LDEB

Behaviour	Genotype		Strain		Interaction	
Transitions	$F_{1,27}$=1.428	P=0.242	$F_{1,27}$=23.425	P<0.001	$F_{1,27}$=16.639	P<0.001
SAPs	$F_{1,27}$=14.248	P=0.001	$F_{1,27}$=38.559	P<0.001	$F_{1,27}$=15.808	P<0.001
Total rears	$F_{1,27}$=5.136	P=0.032	$F_{1,27}$=21.732	P<0.001	$F_{1,27}$=5.104	P=0.032
Rears (dark)	$F_{1,27}$=9.174	P=0.005	$F_{1,27}$=12.720	P<0.001	$F_{1,27}$=0.002	P=0.969
Rears (light)	$F_{1,27}$=0.983	P=0.330	$F_{1,27}$=10.728	P=0.003	$F_{1,27}$=5.795	P=0.023
Grooming (bouts)	$F_{1,27}$=3.980	P=0.056	$F_{1,27}$=9.153	P=0.005	$F_{1,28}$=0.062	P=0.806

Post hoc tests on the number of rears in the light, which showed a main effect of strain and a G x S interaction, did not show differences between the genotypes of each strain. Wildtype MF1 mice performed less than either genotype on the B6 background (+/+, $p<0.001$; −/−, $p=0.005$).

Post hoc tests on the number of grooming bouts, which showed a main effect of strain, revealed that wildtype MF1 mice performed less grooming bouts than $NK1^{-/-}$ mice on the B6 background ($p=0.013$).

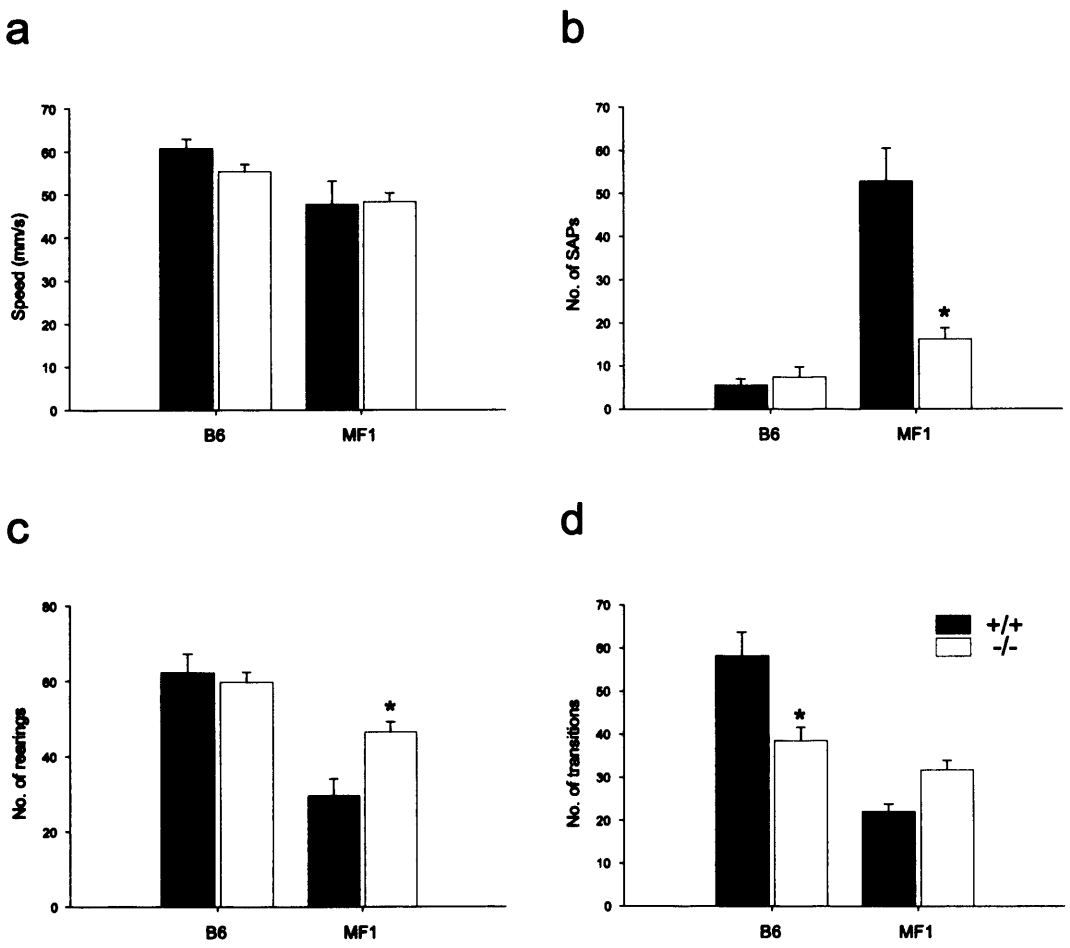

Figure 3.5: Behaviours in the light/dark exploration box. Mean ± SEM of (a) total activity, (b) total rears, (c) stretch-attend postures and (d) transitions in the LDEB. *, $p<0.05$ vs. +/+; n=8.

3.4 *Discussion*

The aim of the experiments in this chapter was to investigate differences between the $NK1^{-/-}$ phenotype produced on two different genetic backgrounds: a mixed MF1 x 129/sv x C57BL/6 hybrid (MF1) and the C57BL/6 backcross (B6). Two behaviours shown previously to be altered by disruption of the NK1 receptor were chosen: morphine-induced hyperlocomotion and behaviour in the light/dark exploration box (LDEB).

3.4.1 *Morphine hyperlocomotion*

Hyperlocomotion in response to many drugs of abuse results from increased dopaminergic input to the striatum and is therefore believed to reflect the motivational processes that underlie aspects of drug addiction (Wise, 1998). The $NK1^{-/-}$ mouse has previously been shown to decrease its locomotor response to morphine at doses which cause an increase in the wildtype (Murtra et al., 2000; Gadd, 2003). Additionally, the rewarding, reinforcing and sensitising properties of the opiates as measured via CPP, self-administration or locomotor sensitisation, respectively, are also attenuated in the $NK1^{-/-}$ mouse (Murtra et al., 2000; Ripley et al., 2002). In this chapter $NK1^{-/-}$ mice on the MF1 background are shown to have an attenuated, but not abolished, locomotor response to morphine. In contrast, there was no difference between the two genotypes when the mutation was transferred onto the congenic B6 background. A further set of experiments confirmed that this was not an effect of dose by showing that in B6 mice there was no difference between genotypes at a series of other doses. Furthermore, analysis of the habituation period showed heightened locomotor activity in $NK1^{-/-}$ mice compared to wildtype in the MF1 but not in the B6 strain.

3.4.2 *Discrepancies with previous work*

Each strain then presents discrepancies with the previous work by Murtra et al. (2000), Ripley et al. (2002) and Gadd (2003), which may be explained by differences in genetic background, experimental conditions and protocol, or a combination of both. Firstly, in the MF1 mice, disruption of the NK1 receptor did not produce a complete abolition of the locomotor response to morphine. Previously, a reduction in speed after morphine compared to saline was found in the $NK1^{-/-}$ mouse by both Murtra et al. (2000) and Gadd (2003). Gadd (2003) observed that the MF1 mice used in that study (and in this thesis) displayed a lower sensitivity to the effects of morphine than those used by Murtra et al. (2000) in both a locomotor stimulating paradigm and CPP experiments. This indicates that some differences are conferred by the change in genetic background.

However, for comparison of the studies other than Murtra et al. (2000), experimental conditions are likely to be important as well. Of these conditions, two key differences to highlight are the amount of habituation, if any, and the doses of morphine examined.

3.4.2.1 *Effect of habituation*

In the experiments in this thesis the animals were habituated to the test arenas for one hour before morphine administration. During this hour the animals were observed to habituate to the boxes by decreasing their activity. As a result the speeds in response to saline injection were very low in all groups and continued to decrease throughout the further two hours recording. This produced a low baseline locomotion over which drug-induced increases were easy to detect. In contrast, in the experiments by Murtra et al. (2000) and Gadd (2003), there was no habituation to the test environment before drug administration. Novel testing environments represent stressful stimuli to rodents even if care is taken to minimize the overtly stressful features such as bright lighting or loud noises (Leussis and Bolivar, 2006). The effects of stress on animals can be unpredictable and depend on many factors. These include the animal's genetic background as well as the effects of drugs. Interactions with the physiological reactions to stress will affect the outcome of any behavioural measures. Stress can have either a hyperlocomotor or a hypolocomotor effect in different situations depending on the nature of the stressor, the species and strain, and the testing conditions used. Stress is also known to affect responses to many drugs among them the psychostimulants (Marinelli and Piazza, 2002), morphine (Stohr et al., 1999; del Rosario et al., 2002), nicotine (Kita et al., 1999) and ethanol (Blakley and Pohorecky, 2006). In behavioural sensitisation paradigms stress can lead to a cross-sensitisation with drugs of abuse (Deroche et al., 1992). Many of these effects are believed to be mediated by corticotrophin releasing factor (Sarnyai et al., 2001). The effect of stress during behavioural experiments will be revisited in other sections of this thesis in more detail.

3.4.2.2 *Effect of dose*

Both Murtra et al. (2000) and Gadd (2003) examined morphine-induced locomotion and CPP at a range of doses. Gadd (2003) used 15 mg/kg as the highest dose tested and although a separation of genotypes was observed with $NK1^{-/-}$ mice decreasing their speed, this dose did not cause an increase in locomotion in wildtype mice. This is in agreement with pilot studies conducted in wildtype mice before the experiments presented here. These showed only a small increase at 15 mg/kg and resulted in the decision to use 20 mg/kg in the later experiments (data not shown). The results of Murtra et al. (2000) are difficult to interpret because the original mixed 129:B6 strain was used, although it has been suggested that the MF1 mice show a reduced sensitivity to morphine compared to the original 129:B6 mice (Gadd, 2003).

$NK1^{-/-}$ mice on both the MF1 and the 129:B6 backgrounds have been shown to develop CPP to morphine at high doses (Murtra et al., 2000; Gadd, 2003). This observation fits with the data presented here showing that the response to morphine is blunted but not abolished completely. Together these data suggest that there may be a shift in the dose response curve to morphine in the $NK1^{-/-}$ mouse in the MF1 strain and there are also differences between wildtype mice on each of these backgrounds (Gadd, 2003). Deletion of the NK1 receptor then does not bring about a complete insensitivity to morphine, an observation supported by the finding that hot-plate analgesia to morphine is intact in the knockout mouse (De Felipe et al., 1998). Furthermore, no differences between morphine metabolism, μ-opioid receptor number, binding or function, receptor mRNA expression and modulation of adenylate cyclase activity by morphine have been found between the genotypes (Murtra et al., 2000; Gadd, 2003). This implies that the shift in morphine sensitivity in the knockout mouse is downstream of the receptor or involves a more complex change in behaviour at a systems level.

3.4.3 *Strain-genotype interactions*

In B6 mice no difference in morphine-induced locomotion was observed between genotypes at any dose tested. A stimulatory effect of morphine was detected after administration of 5 mg/kg indicating a substantial shift in the dose response curve of the drug. This suggests that there is an interaction between some feature of the particular genetic background, the response to opiates and the NK1 receptor. Different genetic backgrounds can result in highly variable sensitivity to pharmaceutical agents due to a number of reasons. Differences in ligand receptor function or number, drug metabolism, and access to target structure as well as complicated epistatic interactions with other genes and regulatory elements can all result in shifted sensitivity to a particular compound (Horowitz and Dudek, 1983). On the other hand, differences in the dose response curve between genetic backgrounds may exist because morphine's effects are mediated by different pathways in each strain. Locomotion is not a simple behavioural output but is a polygenic trait that relies on the integration of a number of different neural substrates (Mink and Thach, 1993; Flint et al., 1995). Although the psychostimulants such as cocaine are known to increase locomotion primarily through increasing striatal dopamine (Wise, 1998), there is evidence that morphine acts on different pathways and can have a variety of effects on both motivational and affective systems (Dockstader and van der Kooy, 2001).

Hence, as well as the mesolimbic projection that mediates motivation, morphine acts at a number of other sites as well. Morphine has important effects on μ opioid receptors within areas involved with the control of the HPA axis and affect, such as the LC and the amygdala. Morphine has been shown to produce anxiolytic effects in the light/dark box paradigm (Costall et al., 1989) and in place preference experiments (Dockstader and van der Kooy, 2001). These and other studies have differentiated between the motivational effects of morphine, which require disinhibition of the dopaminergic cells in the VTA, and its anxiolytic effects mediated by limbic structures via the HPA axis (Costall et al., 1989; Dockstader and van der Kooy, 2001). Both of

these effects are known to produce changes in spontaneous locomotion as tested here, despite being subserved by different neural substrates. Importantly, lesion studies have also indicated that the rewarding effects of morphine are in part mediated by systems other than the motivational pathways (Olmstead and Franklin, 1996).

There is evidence to suggest that the NK1 receptor may be involved in mediating the anxiolytic actions of morphine. Experiments have used the selective neurotoxin saporin-substance P to specifically ablate cells expressing the NK1 receptor in different brain regions. These studies have implicated cells in the amygdala, but not the nucleus accumbens, as being essential for the development and expression of morphine reward (Gadd et al., 2003). Whilst the amygdala is part of the limbic system and is known to modulate anxiety, the nucleus accumbens is a critical site for motivated behaviours. Destruction of the NK1 receptor-expressing cells in the amygdala also influenced anxiety-related behaviour and reduced the hyperlocomotor response to morphine (Gadd et al., 2003). These results suggest that NK1 receptors located in the amygdala are involved in the expression of morphine-mediated behavioural effects via their role in anxiolysis.

In the MF1 and 129 strains which are known to be highly reactive to stressors morphine may preferentially increase their locomotion via its anxiolytic effects that increase exploration of novel environments. However, in strains of mice in which the anxiolytic effect of morphine is less important than the motivational component, the NK1 receptor may not be an important factor in influencing responses to morphine. As such in B6 mice, which display low levels of anxiety in novel environments, morphine may produce its effects primarily via stimulation of the mesolimbic dopaminergic pathway. In B6 mice, morphine is known to cause roughly a two-fold increase in extracellular striatal dopamine as compared to 129 mice (Murphy et al., 2001). Furthermore, in this thesis B6 mice were observed to increase their locomotion at far lower doses of morphine than the other strains tested. As such, B6 mice may be very sensitive to the locomotor stimulating effects of morphine mediated via motivational systems.

More support for the hypothesis that the NK1 receptor is involved in the affective, and not the motivational, properties of morphine comes from work using other drugs. Responses of $NK1^{-/-}$ mice or wildtype mice administered NK1 receptor antagonists have been shown to be unaffected to cocaine (Murtra et al., 2000; Ripley et al., 2002; Placenza et al., 2006). Cocaine is believed to increase locomotion primarily via stimulation of the mesolimbic motivational pathway and also lacks an anxiolytic component (Dockstader and van der Kooy, 2001).

Taken together these results imply that morphine increases locomotion through at least two separate pathways and that the NK1 receptor is critically involved in one of these. Morphine activates these pathways to a differing extent in each strain which may account for the observed differences in the effect of NK1 receptor disruption in each strain.

3.4.4 Differences in basal locomotion

Another significant feature of the results in this chapter was revealed by analysis of the habituation periods of mice. As described above, the studies previously conducted on morphine-induced locomotion in $NK1^{-/-}$ mice differed as they did not use an habituation period (Murtra et al., 2000; Gadd, 2003). In this study, analysis of the habituation period revealed that $NK1^{-/-}$ mice on the MF1 background moved faster than wildtype. The knockout mice also displayed higher locomotion than wildtype following saline injection. In contrast, on the B6 background no difference was found between the genotypes during habituation although if anything there was a trend for wildtype mice to move faster than $NK1^{-/-}$ mice ($p=0.058$). Similarly in the B6 strain after saline injection there was no difference between genotypes. This result implies that in MF1 mice the novel environment has a strain-dependent stimulating effect on locomotion that is greater in $NK1^{-/-}$ mice than wildtype. This result supports the idea that anxiety-related behaviours dependent on the NK1 receptor are of more importance in the MF1 strain than the B6 strain.

3.4.5 *Behaviour in the LDEB*

As well as morphine-induced hyperlocomotion, behaviour in the LDEB was also examined in this chapter. $NK1^{-/-}$ mice on the MF1 background differed from wildtype counterparts on a number of behaviours. Most of these behaviours corresponded with a more exploratory, anxiolytic phenotype. As such, $NK1^{-/-}$ mice performed less risk assessment behaviours and more rears than wildtype mice. On the other hand, $NK1^{-/-}$ mice on the B6 background differed from their wildtype counterparts on only one behaviour, the number of transitions between compartments, in which wildtype mice made significantly more than knockout animals. Increased transitions have been associated by many authors as indicating anxiolytic properties (Bourin and Hascoet, 2003).

The biggest overall effect in the experiment was that of strain. Of the fourteen behaviours measured, eight showed a main effect of strain and four of these behaviours also showed an interaction between strain and genotype.

It has previously been reported that changes in locomotor behaviour can confound the measurement and interpretation of results in the LDEB. As locomotor activity was identified as being dependent on strain, it was factored into the statistical design and removed as a covariate. Following this procedure, all eight behaviours still showed a main effect of strain and the main effect of genotype was gained on total rears but lost on grooming bouts. This demonstrates that differences in locomotion between the strains cannot account for the differences in behaviour measured. The interactions in a number of behaviours between strain and genotype show that knockout of the NK1 receptor can have strain-dependent effects on behaviour in the LDEB.

With a number of the behaviours it is possible that there were no differences between genotypes in the B6 mice because of a floor or ceiling effect. For example, the wildtype B6 mice perform so few SAPs that it is unlikely that the $NK1^{-/-}$ mouse could perform less. Similarly, wildtype B6 mice perform far more rears than MF1 mice and this high rate of responding may obscure any increase due to the mutation. It

could be argued that the B6 mouse was outside the "working range" for many of the behaviours measured in the LDEB suggesting that they would be a poor mouse to use in this test. However, according to many reports B6 mice are one of "the strains of choice for anti-anxiety testing in the light/dark test" due to the robust effect of diazepam on increasing exploratory behaviours (Crawley and Davis, 1982; Bourin and Hascoet, 2003). On the other hand, other studies have failed to show B6 mice displaying anxiolytic behaviour in the light/dark box at any dose (Hascoet and Bourin, 1998; Griebel et al., 2000). Clearly, many differences exist in the parameters and laboratory conditions between different experimenters and these need to be considered carefully, especially for experiments to investigate anxiety and affective disorders.

3.4.6 Different mouse strains in the LDEB

Although many of the commonly used mouse strains have been tested in the LDEB, this is the first study to directly compare these two strains, a hybrid MF1:129:B6 and the B6. Likewise, few studies investigating strain differences have examined as many parameters in the test as were looked at here. The most comprehensive study examining a variety of strains in the light/dark test was conducted by Griebel et al. (2000). They studied nine different strains including B6. Their apparatus included a tunnel between compartments and two parameters were measured, time spent in the light side and number of transitions. B6 mice, which were included in this study, were classified as displaying an intermediate level of anxiety and were not sensitive to the anxiolytic effects of diazepam in this test (Griebel et al., 2000). B6 mice have also been tested in this paradigm by a number of different authors with the general conclusion that they exhibit low anxiety (Crawley and Davis, 1982; Crawley et al., 1997; Hascoet and Bourin, 1998; Lepicard et al., 2000; Rodgers et al., 2002; Carola et al., 2004). One of these studies also examined the behaviour of 129 substrains in the LDEB (Rodgers et al., 2002), due to their importance in the creation of genetically modified animals (reviewed in section 1.2). Although differences in 129 substrains have been noted (Simpson et al.,

1997; Cook et al., 2002), the majority of work supports the notion that in exploration-based anxiety models they exhibit a hypolocomotor response which may compromise some behavioural parameters (Crabbe et al., 1999; Homanics et al., 1999; Contet et al., 2001; Bolivar et al., 2000).

In contrast to B6 and 129 backgrounds, the MF1 strain has been little studied with respect to anxiety-related behaviours. As an outbred strain there is a higher degree of variability in its behaviour than inbred strains. This variability has been used pinpoint RGS2 as a modulator of anxiety using quantitative trait analysis (Yalcin et al., 2004). However, a thorough understanding of how addition of the MF1-derived alleles may contribute to anxiety-related behaviours is lacking from the literature.

In this study, wildtype B6 mice exhibited higher locomotion, exploration (as assayed by rears and transitions) and performed fewer risk-assessment behaviours (SAPs) than the mixed MF1 background, as would be suggested by work comparing the B6 and 129 strains (Rodgers et al., 2002). Even after accounting for hypolocomotion in the MF1 strain by removing it as a covariate many of the behavioural parameters still displayed highly significant strain effects. This indicates an extremely high level of strain dependency for behaviours in this anxiety model, and by inference, for many of the other tests used to assay anxiety-related behaviour in mice. These findings accentuate the need to consider background strain carefully when creating genetically modified mice.

3.4.7 Mutant mice and the LDEB

The LDEB has also been used extensively to study anxiety-related behaviours in genetically modified mice (Bolivar et al., 2000; Belzung and Griebel, 2001). Here, we have shown strain-dependent effects on the anxiety-related behaviour of $NK1^{-/-}$ mice. $NK1^{-/-}$ mice on the MF1 background displayed anxiolytic behaviour whereas knockout mice on the B6 background did not. In concordance with these results, other studies using $NK1^{-/-}$ mice on the MF1 background have detected an anxiolytic

effect of the mutation in the LDEB (Fisher, 2005; Herpfer et al., 2005). Both of these studies implicated the noradrenergic system in these changes. In other animal models of anxiety, an anxiolytic effect has been found in $NK1^{-/-}$ mice and wildtype mice after administration of NK1 receptor antagonists (Rupniak et al., 2001; Santarelli et al., 2001; Bilkei-Gorzo et al., 2002; Santarelli et al., 2002).

The lack of anxiolytic profile in $NK1^{-/-}$ mice on the B6 background could be attributed to their low anxiety profile as highlighted in previous paragraphs. The lack of effect of diazepam in a number of these studies has been attributed to this explanation. Another interpretation of the data is that in the B6 strain the NK1 receptor is not involved in anxiety-related behaviours, at least as measured in the LDEB. Future experiments using more aversive stimuli and tests examining other components of anxiety-related behaviour are required to determine which of these hypotheses is correct.

3.4.8 NK2 and NK3 effects

There is also a body of evidence that implicates the other tachykinin receptors, NK2 and NK3, in behaviours linked to affective disorders. The NK2 receptor antagonists, GR159897 and SR489698, have shown efficacy in a number of animal models of anxiety (Stratton et al., 1993; Walsh et al., 1995; Teixeira et al., 1996; Griebel et al., 2001; Salome et al., 2006). Additionally, SR489698 has also displayed antidepressant-like effects in the forced-swim test in rats (Steinberg et al., 2001; Dableh et al., 2005). Although NK3 antagonists have been less extensively studied, senktide has been observed to decrease anxiety in the EPM (Ribeiro et al., 1999) and SR142801 has produced anxiolytic effects in the social interaction test in gerbils (Salome et al., 2006) and antidepressant-like effects in the forced-swim test in rats (Dableh et al., 2005). Additionally, NK3 receptors in the LC have been shown to be essential for the activation of neurons in this area by NK1 receptor agonists (Bert et al., 2002). The involvement of the other tachykinins and tachykinin receptors in modulating anxiety-related behaviours is another area that

warrants further study.

3.4.9 Summary

The experiments in this chapter have shown that knockout of the NK1 receptor affects both addiction- and anxiety-related behaviours in a profoundly strain-dependent manner. Morphine-induced hyperlocomotion was severely attenuated in $NK1^{-/-}$ mice on the MF1 background. In contrast, when the mutation was backcrossed onto the B6 background the knockout animal was indistinguishable from the wildtype. In the LDEB the $NK1^{-/-}$ mouse on the MF1 background exhibited several behaviours that were indicative of an anxiolytic phenotype. After crossing onto the B6 background the anxiolytic profile was lost but the genotypes were separable by a different trait — decreased number of transitions — normally associated with anxiogenic drugs. In conclusion, these experiments show that complex interactions exist between genetic background and targeted genetic mutations made for experimental purposes. The next chapters explore this finding in more detail.

Chapter 4

Results II: Reintroduction of the 129/sv strain

4.1 *Introduction*

In the previous chapter, a set of experiments demonstrating the strain dependency of the NK1$^{-/-}$ phenotype were described. Many of the key behavioural differences between the genotypes in the MF1 strain were lost on the B6 background making the study of NK1 receptor function difficult, if not impossible, in this mouse. It was therefore decided to reintroduce the 129/sv strain, one known to have higher basal anxiety-like behaviour. By backcrossing over several generations the mutation could be transferred onto this background thereby allowing the production of hybrid F1 mice as recommended by the Banbury Conference (Silva et al., 1997) (see figure 1.1). While this breeding program was in progress the behavioural and molecular phenotype of the intermediate generations of mice was studied to see how the gradual dilution of the B6 background affected their responses. This and the next chapter describe these experiments.

4.2 *Materials & Methods*

The mice used in this and the next chapter were produced by crossing female B6 NK1$^{-/-}$ mice with wildtype male 129/sv mice purchased from Harlan (Bicester, UK).

The resulting heterozygotes on a mixed B6:129 hybrid background were crossed to produce homozygotes and heterozygotes. Either mice from this original cross or the F1 generation of crosses between these homozygotes were used. These mice will be referred to as B6:129 mice to differentiate them from the original 129:B6 mixed strain used by De Felipe et al. (1998) and Murtra et al. (2000). See figure 2.2 for more information.

The morphine-induced locomotor hyperactivity and LDEB experiments were performed as described in the previous chapter. For the morphine hyperlocomotion experiment, after 1 h of habituation mice were administered morphine and had their locomotor activity recorded for the subsequent 2 h. Mice were administered either 0, 10, 20 or 30 mg/kg morphine. For the LDEB experiments after 1 h habituation, animals were placed in the light compartment and behaviour was observed and scored for 10 min.

4.3 Results

4.3.1 Baseline locomotion

Baseline locomotion was assessed as in the previous chapter by analysing the activity of all animals during the habituation period as well as animals that received saline injections during the testing period. Figure 4.1 summarises these results. 1-way ANOVA conducted on speed moved during habituation with genotype as the between subjects factor revealed a main effect of genotype ($F_{1,85}$=14.956, $p<0.001$) showing that $NK1^{-/-}$ mice moved faster than their wildtype counterparts during this period. In contrast, 1-way ANOVA on speed moved after saline injection revealed no difference between the genotypes ($F_{1,14}$=1.041, p=0.325). This result implies that $NK1^{-/-}$ mice may react to a novel environment differently than wildtype mice when the mutation is in the B6:129 strain. This result partly recapitulates what was found in MF1 mice in the previous chapter.

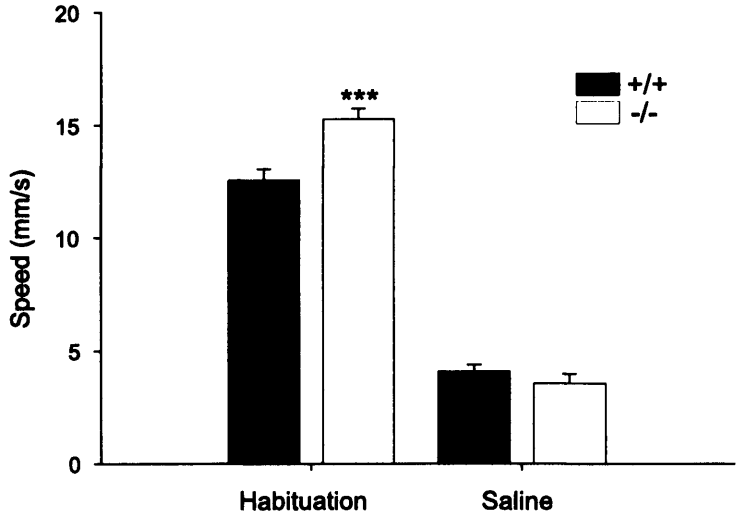

Figure 4.1: Baseline locomotion. Mean ± SEM speed traveled by $NK1^{-/-}$ and wildtype mice on the B6:129 background during the habituation period or after administration of saline. ***, $p<0.001$ vs wildtype; n=7-17.

4.3.2 Morphine-induced locomotion in B6:129 mice

Figure 4.2 shows the locomotor activity induced by different doses of morphine in $NK1^{-/-}$ and wildtype mice on the mixed B6:129 genetic background. 3-way repeated measures ANOVA with genotype (G) and dose (D) as the between subjects effects and time point (T) as the within subjects effect revealed main effects of genotype, dose and time as well as significant G x D, T x D and T x G x D interactions. These results are summarised in table 4.1.

Subsequent 2-way ANOVA on the average speed travelled in the 2 h period following injection with genotype (G) and dose (D) as between subjects factors revealed main effects of genotype and dose and a significant G x D interaction. 1-way ANOVAs for each dose to examine the effect of genotype revealed a main effect of genotype at 20 mg/kg but not at any other dose (figure 4.3).

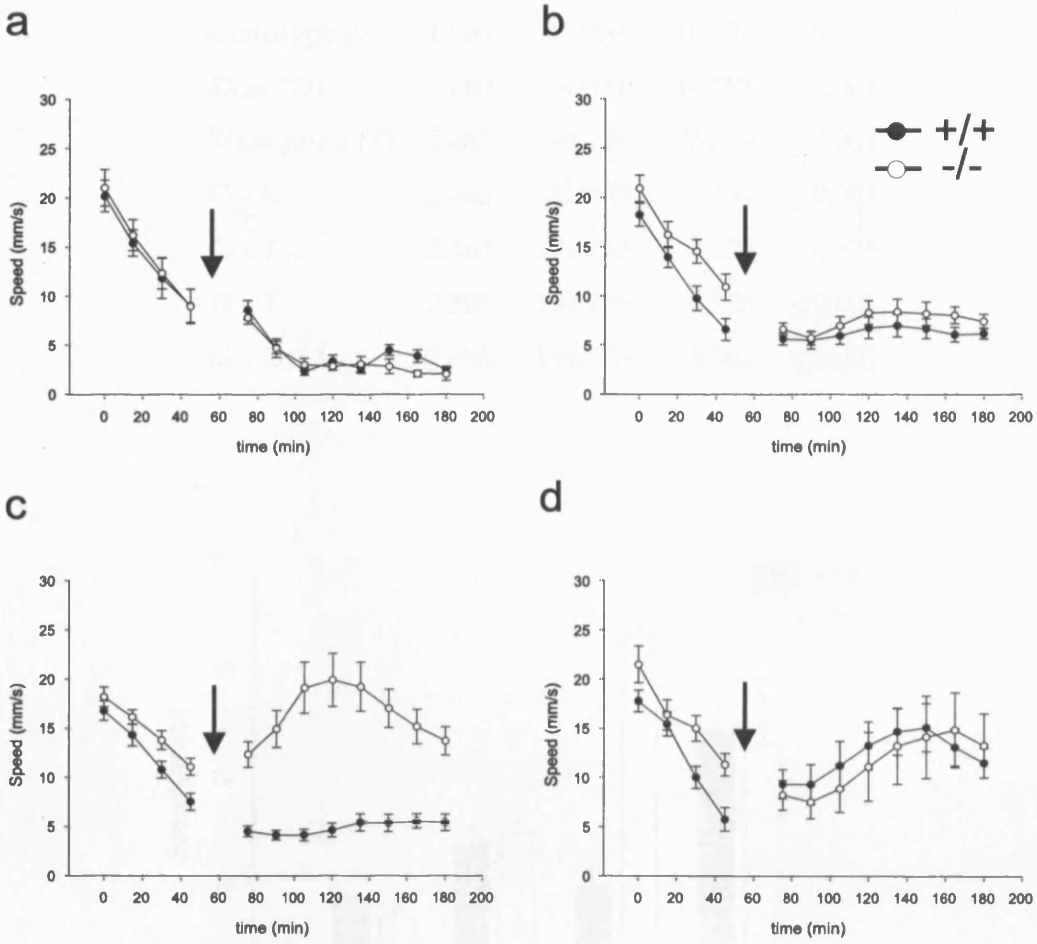

Figure 4.2: Morphine-induced locomotion. Mean ± SEM speed traveled by NK1$^{-/-}$ and wildtype mice on the B6:129 background after administration of saline (a) or morphine (10 mg/kg, b; 20 mg/kg, c; 30 mg/kg, d). Arrows indicate time of injection. n=7-17.

Table 4.1: Results of 3-way ANOVA on morphine-induced locomotor data in B6:129 mice

Factor	d.f.	Error d.f.	F	P
Genotype (G)	1.000	79.000	10.736	0.002
Dose (D)	3.000	79.000	10.788	<0.001
Time point (T)	2.465	194.738	76.856	<0.001
G x D	3.000	79.000	9.150	<0.001
G x T	2.465	194.738	1.123	0.335
D x T	7.395	194.738	9.328	<0.001
G x D x T	7.395	194.738	5.802	<0.001

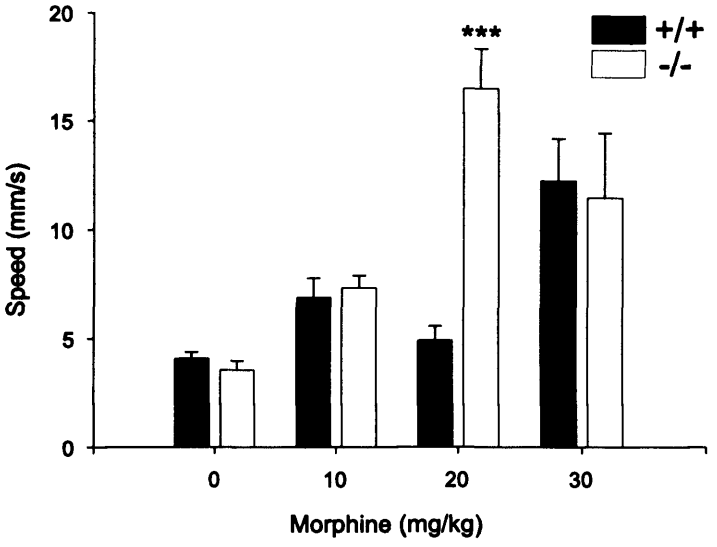

Figure 4.3: Morphine dose response. Mean ± SEM speed traveled by NK1$^{-/-}$ and wildtype mice on the B6:129 background after administration of saline or morphine. ***, p<0.001 vs wildtype; n=7-17.

4.3.3 LDEB in B6:129 mice

$NK1^{-/-}$ and wildtype mice on the mixed B6:129 background were tested in the LDEB as outlined for the MF1 and B6 strains in the previous chapter. All behaviours were scored and values are given in table 4.2 below. A MANOVA test with genotype as the main factor was conducted to control for multiple comparisons. As genotype failed to reach significance ($F_{13,3}$=1.345, p=0.455) further probing of the dataset was precluded although two behaviours, latency to leave the light compartment and number of SAPs, appeared to show a trend that differed between the genotypes. In general, the values obtained for behaviours fell in between those found in the two other strains. These results suggest that crossing B6 mice with 129 mice resulted in a strain with intermediate phenotype. This may have masked the effect of the mutation previously observed in different strains.

Table 4.2: LDEB results in B6:129 mice. Values are mean with SEM in parentheses; n=8-9.

Behaviour	B6:129 +/+		B6:129 -/-	
Latency (s)	23.2	(5.0)	12.0	(2.0)
Return to light	27.3	(14.3)	29.7	(19.8)
Activity (mm/s)	32.5	(2.9)	35.3	(4.4)
Activity:Dark (mm/s)	23.4	(2.1)	28.2	(3.6)
Activity:Light (mm/s)	44.4	(1.5)	45.6	(3.0)
Time:Dark (s)	315.1	(37.2)	272.1	(48.6)
Time:Light (s)	283.7	(37.2)	327.1	(48.5)
Transitions	35.0	(3.9)	38.3	(6.0)
SAPs	15.3	(3.2)	8.2	(2.8)
Rears (Total)	38.5	(6.5)	47.3	(9.6)
Rears (Dark)	10.6	(1.6)	11.6	(2.8)
Rears (Light)	27.9	(5.5)	35.8	(7.2)
Grooming (bouts)	1.3	(0.3)	2.0	(0.3)
Grooming (s)	16.0	(3.1)	20.4	(4.8)

4.4 Discussion

In this chapter the behaviour of a newly created hybrid strain of $NK1^{-/-}$ and wildtype mice was tested. Two protocols, morphine-induced hyperlocomotion and behaviour in the LDEB were examined. In the previous chapter the effect of disruption of the NK1 receptor on these two behaviours was shown to be strain-dependent. As such, the attenuation of morphine-induced hyperlocomotion in $NK1^{-/-}$ mice on the MF1 background was not present when tested on the B6 congenic background. The $NK1^{-/-}$ mouse on the MF1 background also displayed an anxiolytic profile in the LDEB. These behaviours similarly disappeared after crossing onto the B6 background. By crossing back onto the 129/sv background in this chapter it was hoped that the

behaviours associated with the MF1 mouse (and the original 129:B6 mouse) would be recapitulated.

4.4.1 Morphine-induced locomotion

Morphine-induced hyperlocomotion was assessed at a number of different doses in the newly created B6:129 strain. At 10 mg/kg no increase in locomotion compared to saline was observed in either genotype. This result replicates what was found in the MF1 strain. 20 mg/kg produced a significant locomotor activation in the $NK1^{-/-}$ mouse that was not seen in their wildtype counterparts. This result is intriguing as it contrasts with what has previously been found in the MF1 strain and in the original 129:B6 strain. 30 mg/kg of morphine produced an increase in locomotion to the same extent in both genotypes. These results suggest that there is an interaction between the NK1 receptor, an aspect of the opioid system and genetic background that is capable of producing complex effects on the behavioural output. The NK1 receptor appears to be uninvolved in the locomotor response to the opiates in the B6 strain as disruption of the receptor has no effect on this behaviour. In contrast, in the MF1 strain the NK1 receptor appears to be responsible in part for mediating the increase in locomotion caused by morphine as disruption of the receptor causes a major attenuation of this behaviour. Additionally, in the MF1 strain the rewarding, reinforcing and sensitising properties of morphine are attenuated in the $NK1^{-/-}$ mouse and the physical responses to opiate withdrawal are reduced (Murtra et al., 2000; Ripley et al., 2002). Surprisingly, here we find that on a mixed B6:129 background the locomotor response to morphine is increased. It appears that on this background knockout of the NK1 receptor potentiates or sensitises the opioid system to morphine challenge. Although variation in experimental procedure can not be completely discounted, the reasons for this difference most likely stem from differences in the genetic background of the mice.

Differences have been noted, as described in the previous chapter, between the sensitivity to morphine in each of the strains tested. Gadd (2003) observed MF1 mice to

be slightly less sensitive to the rewarding and stimulating properties of morphine than the original 129:B6 mice used by Murtra et al. (2000). Additionally, work in this thesis has shown B6 mice to be far more sensitive than either of these strains, at least to the effects of morphine on locomotion. The B6:129 mouse tested in this chapter displays a relatively low level of locomotion both before and after administration of morphine that is more akin to that observed in the MF1 and the original 129:B6 mice used by Murtra et al. (2000) and Gadd (2003). This is in agreement with studies describing 129 strains as showing hypolocomotion in a number of behavioural tasks (Crabbe et al., 1999; Homanics et al., 1999; Bolivar et al., 2000; Contet et al., 2001).

4.4.2 Strain differences in morphine-induced behaviours

The finding that $NK1^{-/-}$ mice on the B6:129 background appear more sensitive to the locomotor stimulating effects of morphine is surprising and warrants further consideration. Substantial variability exists between the propensity for different individuals to develop addiction to drugs, including the opiates. It should therefore not come as a surprise that variability exists between different mouse strains in this regard. Although there is a large body of work examining many of the mouse strains, there is a paucity of data specifically comparing opiate-mediated behaviours in 129/sv and C57BL/6 mice. This is likely due to 129 substrains performing poorly on the behavioural tests used to assay reward (Miner, 1997). Some studies have been conducted, however, and these may be instructive in helping to explain the results presented in this chapter. One such study has directly compared B6 mice and 129:B6 hybrid mice with regard to heroin-induced conditioned reward and locomotor sensitisation (Szumlinski et al., 2005). Here, B6 mice were found to develop a strong place preference to heroin that was reversed to a place aversion in B6:129 mice. As the hybrid mouse was backcrossed onto the B6 background this aversion was in turn reversed back to a preference in a strain dose-dependent manner. Furthermore, locomotor sensitisation was only observed in B6 mice whilst B6:129 hybrids and

intermediate generations with varying amounts of B6 strain displayed a reduction in locomotion after repeated administration of heroin. Although only one dose of heroin was tested in these studies, the result is of interest with regard to the differences in morphine-induced locomotion reported in this chapter.

In the study by Ripley et al. (2002), $NK1^{-/-}$ mice were shown to have reduced responding to morphine in self-administration studies. In contrast, other groups have shown that NK1 receptor antagonists will increase self-administration of heroin in rats (Placenza et al., 2006).

These differences, as well as the ones reported in this chapter, likely arise due to differential sensitivities to morphine in the different strains or genotypes. The rewarding and reinforcing properties to morphine may rely on changes in the specific neural circuitry underlying these behaviours or may instead reflect different distributions, expression patterns or functional activities of the opioid receptors.

There is evidence from a number of sources that differences at a cellular level may account for the variability in responses observed here. Genetically, there is a divergence between 129 and B6 mice in the intronic sequences surrounding exons 2 and 3 of *Orpm*, the gene encoding the μ-opioid receptor. These regions may regulate transcript stability and C-terminal splicing (Zhou et al., 2001). Quantitative trait loci mapping has identified regions of Chromosome 10, near *Orpm*, as being important for oral morphine preference (Berrettini et al., 1994). The same QTL has also been identified as being important for other opiate-related traits such as morphine analgesia and [^{3}H]naloxone binding (Belknap et al., 1995). There is also considerable evidence that differences exist in μ opioid receptor expression levels between mouse strains. It is thus possible that interstrain variability in the genomic sequence comprising of or surrounding *Orpm* gives rise to differences in receptor expression, distribution and/or function and that in turn this could lead to unique patterns of opiate-mediated behaviour in each strain. Interestingly, a polymorphism in the gene encoding the human μ opioid receptor may also be linked with opiate-dependence in the human population (Bond et al., 1998).

In the 129/SvEv substrain morphine tolerance does not develop either to implanted

pellets or repeated injections, an effect that may be mediated by an NMDA receptor defect (Kolesnikov et al., 1998). As well as opiate reward-mediated behaviours, there are well-characterised pain-related phenotypic differences observed for the B6 and 129 backgrounds as well as many other mouse strains (Mogil, 1999). Interestingly however, strains of mice that show high sensitivity to opiates in one particular test do not necessarily show high sensitivity on other tests. This is the case for B6 mice which have a low sensitivity to morphine analgesia but are highly sensitive to other opioid-mediated phenomena, including locomotor activation, learning and memory, and muscular rigidity (Mogil et al., 1996). This suggests a high degree of interaction with other components of the nervous system and implies that differences may well exist in patterns of receptor expression as well as absolute levels. This may be important for explaining the genotype-dependent results observed in this thesis as interactions between the NK1 receptor and the μ opioid receptor may occur in several brain regions and this may vary between strains.

4.4.3 Interactions between NK1 and opiate receptors

The NK1 receptor and the opiate receptors are both expressed in the regions of the limbic system such as the amygdala, hypothalamus and the LC as well as in the dorsal horn of the spinal cord (Gadd, 2003; Mansour et al., 1987; Moyse et al., 1997; Chen et al., 2000). *In vitro* experiments have shown heterodimerisation of the NK1 and μ opioid receptor which may lead to alterations in internalisation (Pfeiffer et al., 2003) and PPT-A, the precursor protein to substance P has been shown to regulate trafficking of the δ opioid receptor in the spinal cord (Guan et al., 2005). Additionally, in mice lacking μ opioid receptors, substance P mRNA expression is reduced in the ventromedial hypothalamus (Yoo et al., 2005) and morphine also upregulates NK1 receptor mRNA and protein in cultured cortical neurons (Wan et al., 2006).

Furthermore, it is also unknown at present whether there is interstrain variability in NK1 receptor expression levels or distribution. Although immunocytochemical

techniques suggest that there are no gross differences between the strains studied here, this question has not been addressed in a systematic and quantitative manner. Interestingly, another QTL discovered to influence oral morphine drinking was located on Chromosome 6, in the region of the gene encoding the NK1 receptor. As with the work investigating *Orpm*, it may be that differences in this region between the strains account for the behavioural variability in response to morphine and after disruption of the receptor (Berrettini et al., 1994). Behaviourally, animals heterozygous for the functional NK1 receptor are indistinguishable from wildtypes indicating that the gene is haplosufficient (Santarelli et al., 2001). This would argue against an explanation involving expression levels of the receptor. However, it may be that there are changes in the functional activity or subcellular distribution of the receptor between different strains that are important.

As well as interstrain differences in or around the sequences of the genes that may be directly involved in the changes seen in these mice there is also evidence for differences in regulatory elements that may be distal to these genes (Padjen et al., 2005). Such elements can include enzymes and transcription factors which act to modify, either specifically or otherwise, the expression of genes of interest. Such changes may affect the methylation state of important genes. Methylation of regions of DNA is one method of repressing their expression by changing the chromatin structure (Tsankova et al., 2006). The genes responsible for this methylation can often be passed down via epigenetic mechanisms (Weaver et al., 2004; Mager and Bartolomei, 2005). Work has shown that B6 mice may differ from other strains in the methylation patterns of their DNA making it more resistant to modifications (Schumacher et al., 2000; Padjen et al., 2005).

Other studies using different drugs of abuse have found strain-genotype interactions when examining transgenic mice. Notably, an effect of genetic background was reported for mice over-expressing the 5-HT_3 receptor. The mouse was originally characterised on a B6SJL/F2 background. A phenotype that included reduced alcohol drinking preference, attenuated cocaine place preference and increased cocaine-

induced locomotor stimulation, was found in the transgenic mouse (Engel and Allan, 1999; Allan et al., 2001). However, to investigate the influence of the parental strains, the transgene was bred onto two backgrounds known to differ on a number of these behaviours, the B6 and DBA/2J (D2) strains. Mutant mice on the B6 strain recapitulated the phenotype originally seen. In contrast, the effect of the transgene was only observed in one test when using the D2 mice (Metz et al., 2006). Another study investigating the role of the dopamine D_2 receptor in ethanol-induced stimulation also showed an epistatic interaction between deletion of the gene and background strain, this time between B6 and 129 mice (Palmer et al., 2003). Furthermore, differences in the percentage of B6-derived genes have been shown to confer differing susceptibility to cocaine in a study using a self-administration paradigm (Ruiz-Durantez et al., 2006).

Together these results suggest that genetic background can have a substantial effect on the sensitivity of an animal to specific properties of a drug, which may reflect changes at a molecular, cellular or systems level. Additionally, there are likely to be important interactions between the NK1 receptor and opiate receptors which also contribute to an animal's sensitivity to morphine as well as the particular behavioural consequences this has.

4.4.4 Behaviour in the LDEB

Previously, in this study and others, $NK1^{-/-}$ mice on the MF1 background have been shown to have an anxiolytic profile in the LDEB (Fisher, 2005; Herpfer et al., 2005). This anxiolytic profile was absent in $NK1^{-/-}$ mice on the B6 background, however, either due to being masked by the strain's low baseline anxiety or due to an uninvolvement of the NK1 receptor in anxiety-related behaviours on this background. In this chapter, crossing back onto a 129/sv background to produce a mixed B6:129 mouse failed to uncover any major differences between the genotypes. Two behaviours associated with anxiolysis displayed a trend towards increasing in the knockout but neither reached significance. Overall, however, the phenotype of this mouse appeared intermediate

with respect to the MF1 and B6 strains. The original studies using the NK1$^{-/-}$ mouse on a mixed 129:B6 background by De Felipe et al. (1998) also failed to show an anxiety-related phenotype using the EPM indicating that this particular mix of strains may not be ideal for studying these phenomena. In work using an NK1$^{-/-}$ mouse on a congenic 129 background by Santarelli et al. (2001) an anxiolytic effect in the EPM was observed. 129 strains, as discussed, show a high baseline anxiety and are thought to be highly "emotional". Taken together these results imply that the effect of NK1 receptor disruption on anxiety-related behaviour is more likely to be observed in highly "emotional" strains than others.

It would be predicted from this work that continuing the backcrossing strategy onto a congenic 129 background would eventually, after a number of generations, result in a return of the anxiolytic profile in the NK1$^{-/-}$ mouse. Similarly, as suggested in the previous chapter, the use of more aversive stimuli or different testing procedures may allow the effect of the receptor knockout to be observed.

4.4.5 Summary and conclusions

In summary, the results in this chapter show that crossing the mutation in the NK1 receptor back onto the 129/sv background over one generation leads to the unmasking of an interesting phenotype. Disruption of the receptor caused the knockout mouse to become more sensitive to the locomotor stimulating properties of morphine but had no effect on behaviour in the LDEB. These results again underscore the need to consider the complications introduced by strain selection on experiments using genetically manipulated mice. The next chapter explores differences between these two strains at a molecular level with particular emphasis on the hypothalamic-pituitary-adrenal (HPA) axis.

Chapter 5

Results III: The HPA axis in the NK1-/- mouse

5.1 *Introduction*

The previous two chapters detail a set of experiments comparing the behavioural responses of $NK1^{-/-}$ mice on three different genetic backgrounds. Much of the phenotype caused by the NK1 receptor mutation on the MF1 background was lost after crossing onto a congenic B6 background. After crossing back onto the 129/sv background for one generation a complex phenotype emerged. The differences in baseline locomotion found in the MF1 strain were recapitulated. However, although a genotype-dependent difference in morphine-induced hyperlocomotion was uncovered it opposed the previous findings in the MF1 (and original mixed 129:B6) strain. Behaviours measured in the LDEB seemed for the most part to take on an intermediate phenotype between the wildtype B6 and MF1 strains and although two behaviours associated with anxiolysis showed a trend towards increasing in the knockout they did not differ significantly. This intriguing set of results is the effect of the complex set of interacting genetic factors present in each strain. The consequences of manipulating a single gene are subject to a different set of modifying factors within each genome resulting in unpredictable phenotypic variation. This is not unprecedented and has been found in other studies using transgenic mice. Many of these studies identified changes in the HPA axis between different mouse strains as contributing to observed

variability (Peinado et al., 2005; Carter, 2006). We therefore hypothesised that differing responsivity of the HPA axis may account for some of the inter-strain variation in the NK1 receptor mutant. This chapter examines a number of features of this HPA axis in the $NK1^{-/-}$ mouse on the B6 and the 129 background.

5.1.1 The hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis is responsible for orchestrating the stress-responsive endocrine system via a complex series of connections and feedback loops. Anatomically, as suggested by it's name, it consists of the hypothalamus, the pituitary gland and the adrenal glands. Its main function is to control levels of circulating glucocorticoids which fluctuate in response to external environmental stressors as well as due to sleep/wake patterns (Herbert et al., 2006). Corticotrophin releasing factor (CRF) and arginine vasopressin (AVP) are synthesised in the paraventricular nucleus of the hypothalamus (PVN) and released by nerve terminals into the median eminence. They are transported through the portal blood vessel system of the hypophyseal stalk to the anterior lobe of the pituitary where they stimulate release of adrenocorticotropic hormone (ACTH). ACTH is transported in the blood to the adrenal cortex of the adrenal gland where it stimulates biosynthesis of glucocorticoids from cholesterol (Pariante, 2006). For a summary see figure 5.1. These glucocorticoids, corticosterone in rodents and cortisol in man, have many effects throughout the body, including within the brain. There are two main receptor types: mineralocorticoid or Type I receptors (MR) and glucocorticoid or Type II receptors (GR). They differ in their affinity for corticosterone such that MR have high affinity and so are fully occupied at low hormone levels while GR have far lower affinities and so only become activated upon greater release of corticosterone (Korte, 2001).

GRs can have direct effects on intracellular molecules or can act as transcription factors controlling gene regulation. They also form part of a negative feedback loop in the brain whereby their activation acts to terminate further corticosterone release. This

action is important for coupling the physiological response to a stressor to hormonal release (Korte, 2001). Although release of corticosterone is essential for many processes including the ability to respond to environmental threat, prolonged periods of high release can be harmful (Korte et al., 2005; Herbert et al., 2006). Depression has been linked to disturbances in control of the HPA axis as described in section 1.4.3 of Chapter 1.

5.1.2 *Interactions between the HPA axis and behaviour*

Differences in corticosterone levels and HPA axis activity may account in part for some of the behavioural differences observed in this thesis. The HPA axis is known to impact upon the locomotor response to morphine (Marinelli et al., 1994; Deroche et al., 1995; Stohr et al., 1999) and behaviour in the LDEB (Bourin and Hascoet, 2003), which have been examined in the two previous chapters.

5.1.2.1 *The opiate system and the HPA axis*

The opiate system and the HPA axis interact at a number of different sites. μ opioid receptors are expressed in several areas, such as the LC, the hypothalamus, the hippocampus and the amygdala, which are all known to modulate HPA activity. Opiates affect the HPA axis indirectly via actions on the noradrenergic system (Sarnyai et al., 2001). These effects can be complex, probably due to the wide distribution of opiate receptors, and as such systemic morphine has been shown to both stimulate and inhibit corticosterone release in unstressed and stressed rats, respectively (Suemaru et al., 1989). *In vitro* experiments have shown that direct stimulation of hypothalamic opioid receptors inhibits CRF release (Buckingham, 1982). In turn, glucocorticoids also have a variety of effects on opiate-mediated behaviours such as morphine-conditioned place preference (Ferguson et al., 2004), hyperlocomotor effects (Deroche et al., 1992) and analgesia (Mousa et al., 1981). Long term opiate exposure also inhibits neurogenesis (Eisch et al., 2000).

5.1.2.2 *Anxiety-related behaviours and the HPA axis*

Behaviour in the LDEB assays the effects of a novel environment on animal behaviour. Novel environments represent mild stressors to rodents and so behaviour in this test is also critically linked to activation of the HPA axis (Costall et al., 1989; Bourin and Hascoet, 2003). More work is required in this area to understand why different strains react to testing conditions in different ways and how activation of the HPA axis is involved.

5.1.3 *Neurogenesis*

For many years it was believed that neurogenesis, the formation of new neurons, occurred only during foetal development. However, it is now known to persist throughout adulthood in at least two discrete regions of the brain: the hippocampus, where neurons are born in the subgranular zone of the dentate gyrus, and the olfactory bulbs, which neurons migrate to from the subventricular zone. It has also been observed in the neocortex and striatum, albeit controversially (Gould and Tanapat, 1999). In the olfactory bulb it is believed that new neurons are simply replacing old ones. In the hippocampus, however, the functional role of neurogenesis is less well understood. The addition and assimilation of new neurons into existing circuits would be postulated to enhance hippocampal function. Indeed, suppression of neurogenesis produces memory impairments in hippocampus-dependent trace conditioning (Shors et al., 2001). Similarly, training in a hippocampus-dependent task results in a increase in the number of newly-generated adult granule cells (Gould et al., 1999) and long-term memory of water maze learning shows a requirement for neurogenesis (Snyder et al., 2005). Furthermore, genetic differences in neurogenesis between mouse strains also correlate with acquisition of water maze learning (Kempermann and Gage, 2002). Evidence is therefore accumulating that neurogenesis may contribute to learning and memory processes. Recent work highlights ways in which newly born neurons may contribute to information storage via their NMDA receptors (Tashiro et al., 2006) and

may be involved in episodic memory storage (Aimone et al., 2006).

The role of hippocampal neurogenesis in depression is less well known. As detailed in section 1.4.3 support for the hypothesis linking the two together has come mostly from the investigation of antidepressant effects. The actions of the HPA axis on neurogenesis also provide another causative link. Elevated HPA axis activity, which is associated with depression, has a inhibitory effect on neurogenesis (Cameron and Gould, 1994). The effect of increased circulating glucocorticoids in inhibiting cell proliferation is likely mediated via NMDA receptors (Gould et al., 1994; Cameron et al., 1995). Acute or chronic exposure to stressors in many species also decreases neurogenesis (Gould et al., 1997).

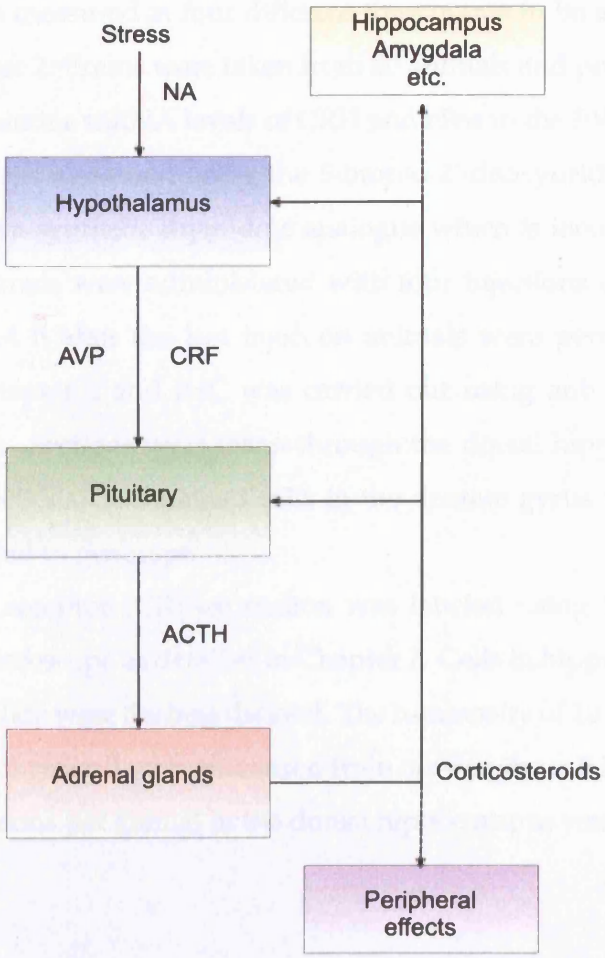

Figure 5.1: The HPA axis. Stress causes the release of noradrenaline (NA) and other peptides which stimulate the release of corticotrophin releasing factor (CRF) and arginine vasopressin (AVP) from the hypothalamus. These act synergistically in the anterior pituitary to produce adrenocorticotropic hormone (ACTH) which is transported via the blood stream to the adrenal glands where it stimulates production of corticosteroids from cholesterol. These corticosteroids have a number of peripheral and central effects and also act as a negative feedback to terminate the stress response via actions in the hippocampus and hypothalamus.

5.2 Materials & Methods

Corticosterone was measured at four different time points in B6 and 129:B6 strains as described in Chapter 2. Brains were taken from all animals and processed using *in situ* hybridisation to examine mRNA levels of CRH and cFos in the PVN.

Neurogenesis was measured using the 5-bromo-2'-deoxyuridine (BrdU) labelling technique. BrdU is a synthetic thymidine analogue which is incorporated into newly divided cells. Animals were administered with four injections of BrdU (50 mg/kg; i.p.) 2 h apart. 24 h after the last injection animals were perfused as detailed in Section 2.1.6 in Chapter 2 and IHC was carried out using anti-BrdU antibody and the DAB technique. Sections were taken through the dorsal hippocampus every 120 μm (approx. 10 sections) and stained cells in the dentate gyrus were counted by an experimenter blinded to genotype.

Glucocorticoid receptor (GR) expression was labeled using IHC and quantified using a confocal microscope as detailed in Chapter 2. Cells in hippocampal CA1 region were measured as they were the best defined. The luminosity of 10 cells per section was measured and a background score measured from outside the cell layer was subtracted from these. 5-6 sections per animal in the dorsal hippocampus were measured.

5.3 Results

5.3.1 Plasma corticosterone

To investigate differences in HPA axis regulation between the strains and genotypes, the corticosterone response to a stressor was measured. 30 min of restraint in a Falcon tube was used as the stressor. 4 treatment groups were examined: no stress; 30 min restraint; 30 min after termination of restraint; and 60 min after termination of restraint. Figure 5.2 summarises this data. 3-way ANOVA with genotype (G), strain (S) and treatment (T) as between subjects factors revealed a main effect of strain and treatment and a significant S x T interaction. The results of this ANOVA appear in table 5.2.

Table 5.1: Plasma corticosterone levels. Mean value in ng/μl with SEM in parentheses. 30 min, immediately after 30 min restraint stress; 60 min and 90 min, 30 min and 60 min, respectively, after termination of 30 min restraint stress. N=8.

Treatment group	B6 +/+		B6 -/-		B6:129 +/+		B6:129 -/-	
Basal	16.1	(3.4)	15.0	(4.3)	13.7	(2.4)	20.9	(4.1)
30 min	257.0	(18.9)	274.7	(15.1)	354.4	(26.1)	347.9	(31.3)
60 min	158.4	(15.0)	185.8	(14.2)	323.1	(52.8)	242.4	(17.4)
90 min	79.5	(23.9)	88.8	(11.3)	152.9	(13.6)	196.8	(32.4)

Subsequent 2-way ANOVAs were conducted on each treatment group with genotype and strain as between subjects factors and are shown in table 5.3. Treatment groups with significant main effects or interactions were further analysed with 1-way ANOVA using group as a between subjects factor and *post hoc* Tukey tests to identify specific differences.

The basal levels of corticosterone showed no effects of either genotype or strain or a significant interaction implying that resting corticosterone levels are not modulated by NK1 receptor knockout or differences between the strains.

2-way ANOVAs on CORT levels in other treatment groups (30 min restraint; 30 min after restraint; 60 min after restraint) revealed a main effect of strain but no effect of genotype and no significant interactions. Data were collapsed across genotypes and 1-way ANOVAs with strain as the main factor revealed a main effect of strain after 30 min restraint ($p=0.001$) and 30 min ($p=0.001$) and 60 min after termination of restraint ($p<0.001$). In other words, after being subjected to a stressor, mice on the B6:129 background released higher levels of corticosterone compared to B6 mice. Although both strains exhibited elevated levels above basal for the following hour the 129:B6 levels were significantly higher than B6. These effects were independent of genotype.

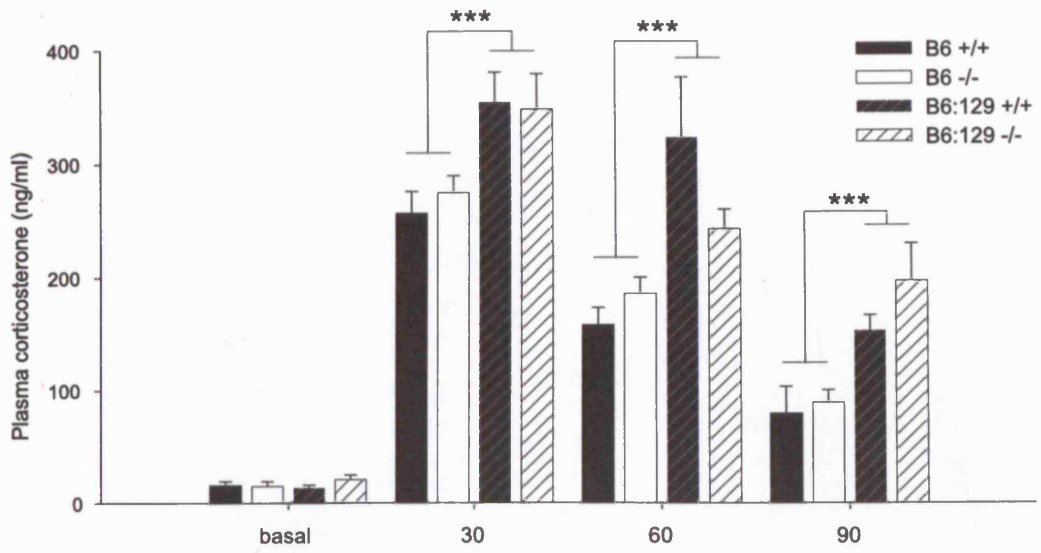

Figure 5.2: Corticosterone levels in B6 and B6:129 mice. Mean ± SEM plasma corticosterone levels basally, after 30 min restraint stress and 30 min and 60 min after termination of restraint stress in B6 and B6:129 strains. N=8. ***, $p<0.001$ B6 vs 129.

Table 5.2: Results of 3-way ANOVA on corticosterone data

Factor	d.f.	Error d.f.	F	P
Genotype (G)	1	112	1.354	0.247
Strain (S)	1	112	28.631	<0.001
Treatment (T)	3	112	270.997	<0.001
G x S	1	112	0.015	0.902
G x T	3	112	0.574	0.634
S x T	3	112	3.721	0.014
G x D x T	3	112	1.425	0.239

Table 5.3: 2-way ANOVAs on corticosterone levels

Treatment group	Genotype		Strain		Interaction	
Basal	$F_{1,28}$=0.693	P=0.412	$F_{1,28}$=0.232	P=0.634	$F_{1,28}$=1.298	P=0.264
30 min	$F_{1,28}$=0.055	P=0.816	$F_{1,28}$=12.942	P=0.001	$F_{1,28}$=0.260	P=0.614
60 min	$F_{1,28}$=0.018	P=0.894	$F_{1,28}$=15.371	P=0.001	$F_{1,28}$=2.585	P=0.119
90 min	$F_{1,28}$=1.542	P=0.225	$F_{1,28}$=17.085	P<0.001	$F_{1,28}$=0.132	P=0.719

5.3.2 *Neurogenesis*

Neurogenesis was measured using the BrdU-labelling technique. The photomicrographs in figure 5.3 show neurogenesis occurring in the dentate gyrus. At higher magnification it is possible to see clusters of dividing cells. All counting was performed at this higher magnification. The data are summarised in table 5.4. Because the different strains were processed at different times no comparisons of strain effects have been made. Unpaired student's t-tests have been used to compare genotypes for both strains and data have been normalised to the wildtype mean for plotting in figure 5.3. Statistical analysis revealed that there was no difference in neurogenesis between the genotypes in the B6 strain (p=0.903). In contrast, in the B6:129 strain $NK1^{-/-}$ mice exhibited significantly more neurogenesis than wildtype mice (p=0.015).

5.3.3 *Glucocorticoid receptor*

GR expression in the hippocampus was measured using IHC as described. The specificity of the antibody was determined using immunoblot analysis as shown in section 2.2. Strong expression of GR was seen in many subfields of the hippocampus with the most clearly labelled cells in CA1 region. Photomicrographs of GR expression are shown in figure 5.4. Unpaired Student's t-tests were used to compare the genotypes in both strains. No difference was found in GR expression between wildtype and $NK1^{-/-}$ mice in the B6 strain (p=0.899). In contrast, in the B6:129 strain GR expression was significantly increased by approximately 40 % in the $NK1^{-/-}$ mouse compared to

Table 5.4: Neurogenesis data. Mean number of cells per section with SEMs in parentheses. N=6-8.

Strain	$NK1^{+/+}$		$NK1^{-/-}$	
B6	22.8	(1.9)	22.5	(1.6)
B6:129	15.9	(1.6)	23.8	(2.4)

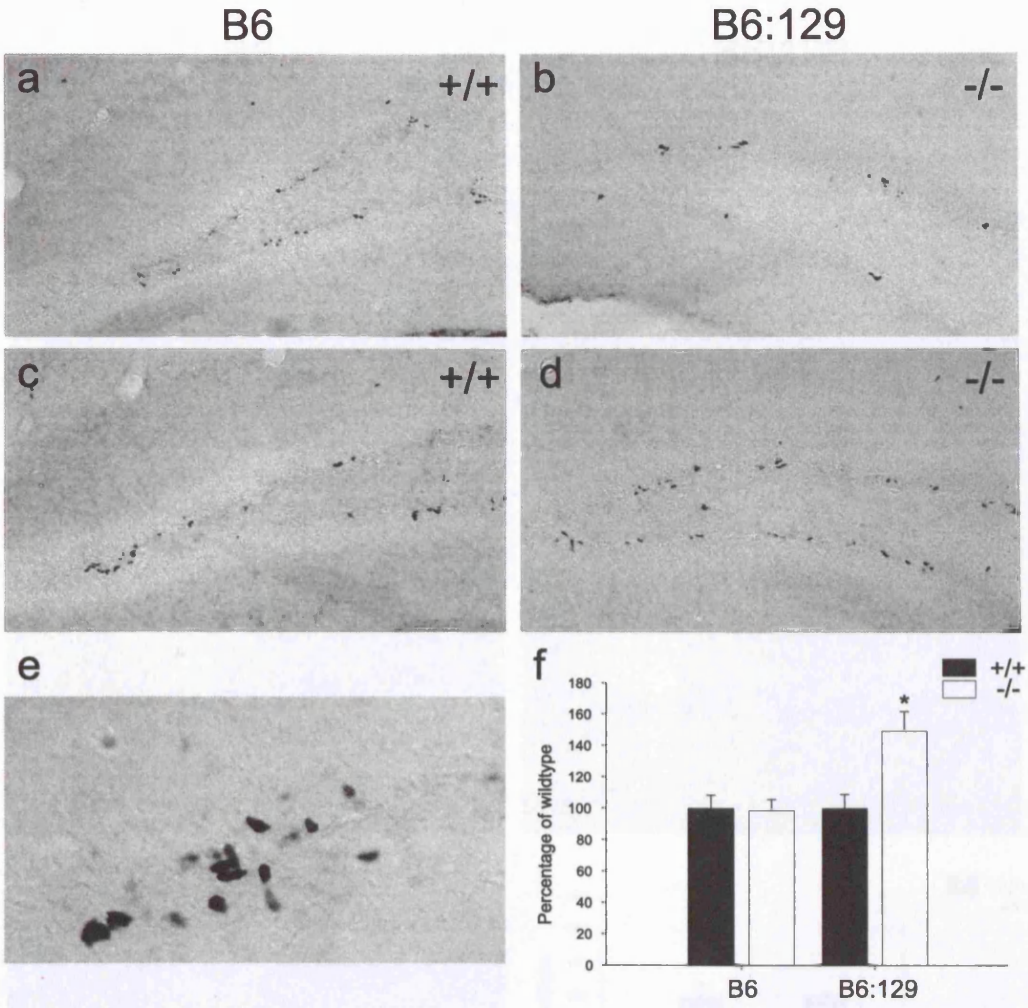

Figure 5.3: Neurogenesis in B6 and B6:129 mice. Photomicrographs show representative examples of BrdU-labelled cells in wildtype and NK1$^{-/-}$ mice on the B6 (a,c) and the B6:129 (b,d) backgrounds. Clusters of cells can be seen dividing at higher power (e). Data are plotted as mean $\pm$ SEM (f). *, $p<0.05$ vs. +/+; N=6-8.

wildtype ($p=0.008$).

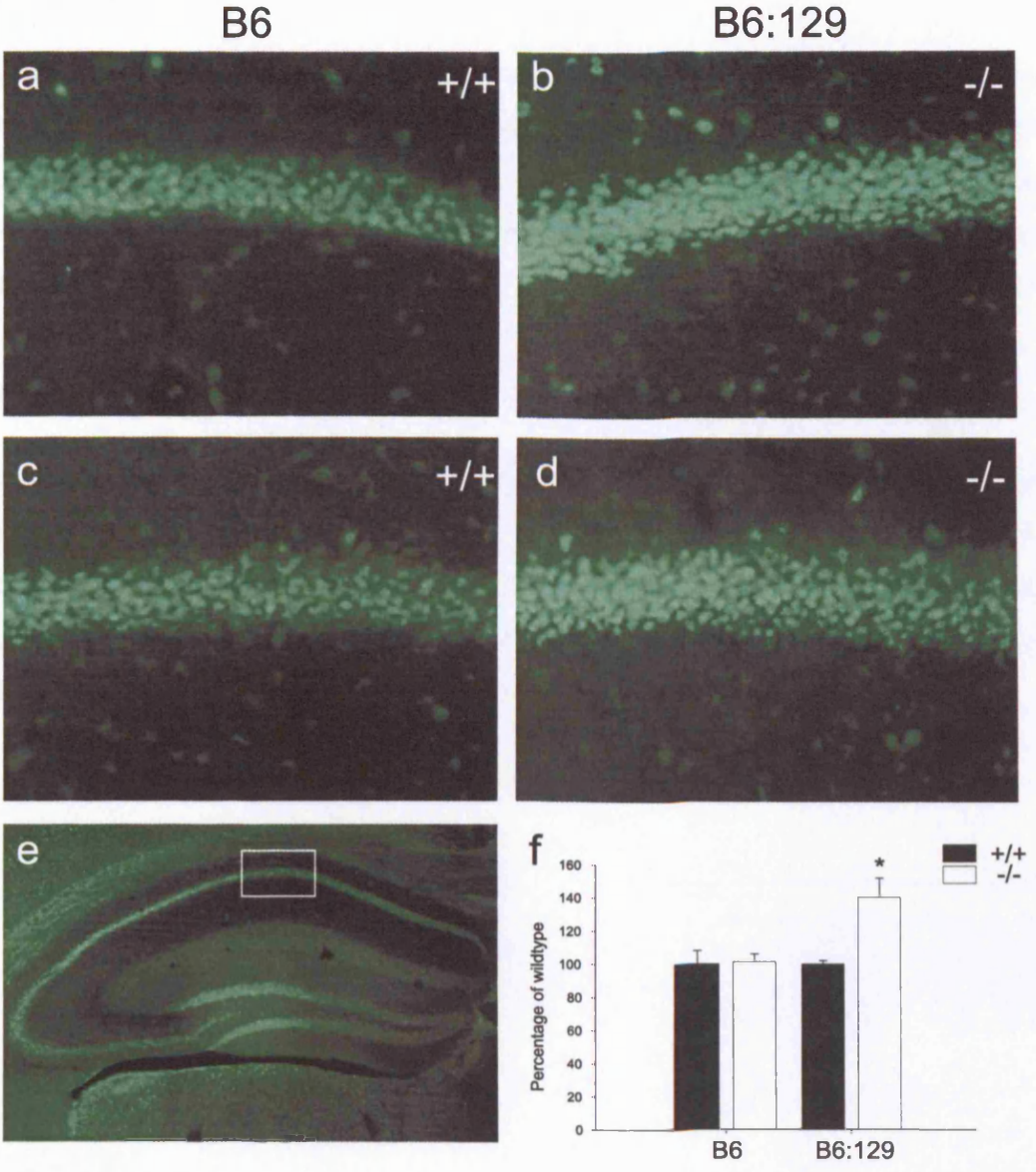

Figure 5.4: Glucocorticoid receptor expression in B6 and B6:129 mice. Photomicrographs show representative examples of GR-labelled cells in wildtype and NK1$^{-/-}$ mice on the B6 (a,c) and the B6:129 (b,d) backgrounds. The whole hippocampus is shown at low power with the equivalent region of CA1 marked by white box (e). Data are plotted as mean $\pm$ SEM (f). *, $p<0.05$ vs +/+; N=6-8.

5.3.4 *CRF*

CRF mRNA levels were measured in the PVN of animals using *in situ* hybridisation. Strong labelling of the PVN was seen that was specific to this region. Figure 5.5 shows example images and a summary of the data. Unpaired student's t-tests were used to compare the genotypes in both strains. No significant differences were found in either B6 (P=0.651) or B6:129 (P=0.538) strains.

5.3.5 *cFos*

cFos mRNA was measured via *in situ* hybridisation in the PVN of animals that had undergone 30 min restraint stress. A discrete activation of cFos was observed in the PVN compared to control animals which had not undergone stress (figure 5.6). Unpaired Student's t-tests were used to compare the genotypes in both strains. No differences were found in either B6 (P=0.977) or B6:129 (P=0.733) strains.

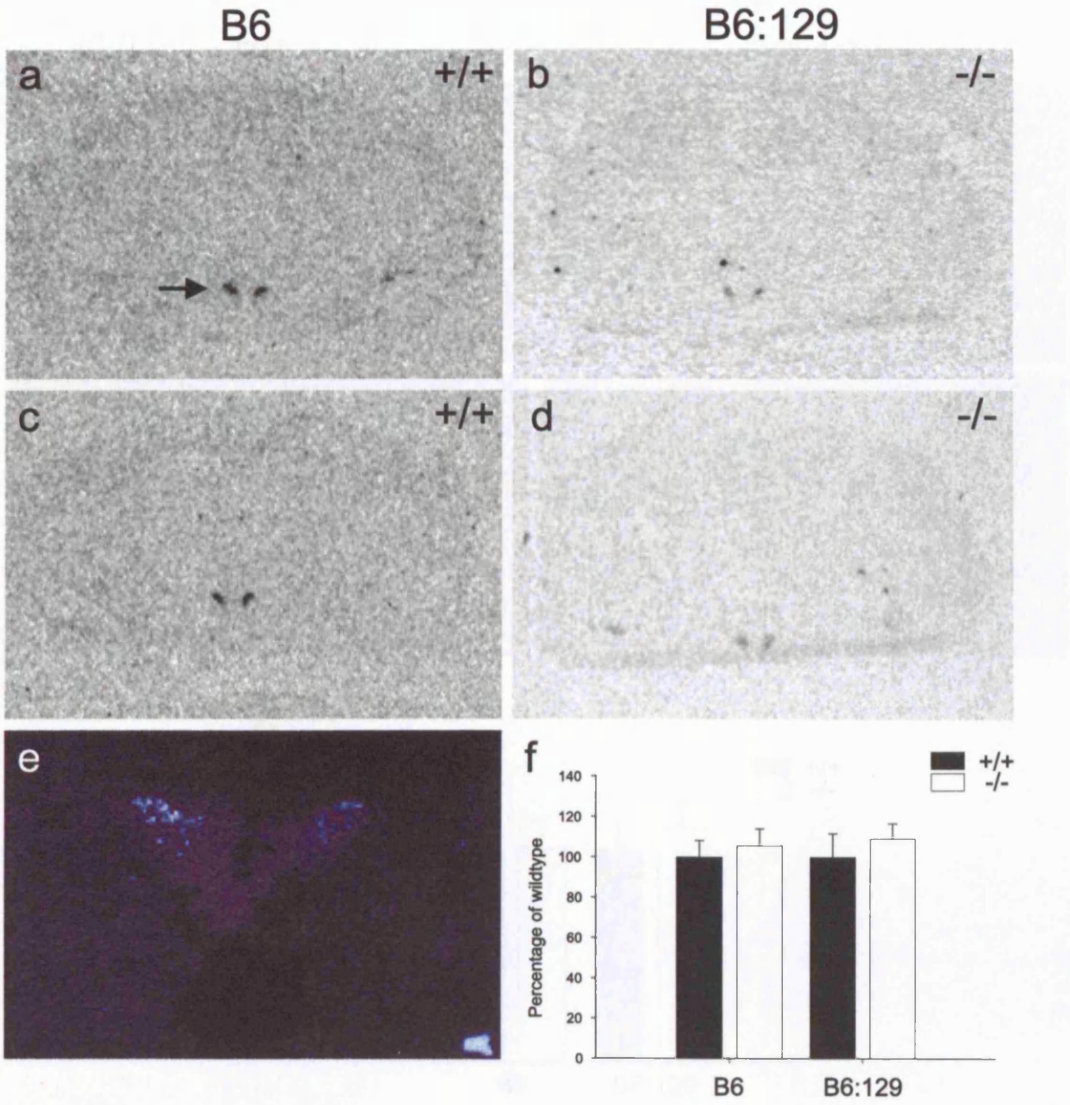

Figure 5.5: Basal CRF mRNA in the PVN (indicated by arrow in a) in B6 and B6:129 mice. Photographs show representative examples of brain sections labelled with ^{35}S-CRF in wildtype and NK1$^{-/-}$ mice on the B6 (a,c) and the B6:129 (b,d) backgrounds. Specific expression is seen in the PVN as shown by this example of darkfield emulsion-dipped section (e). Data are plotted as mean $\pm$ SEM (f). N=4.

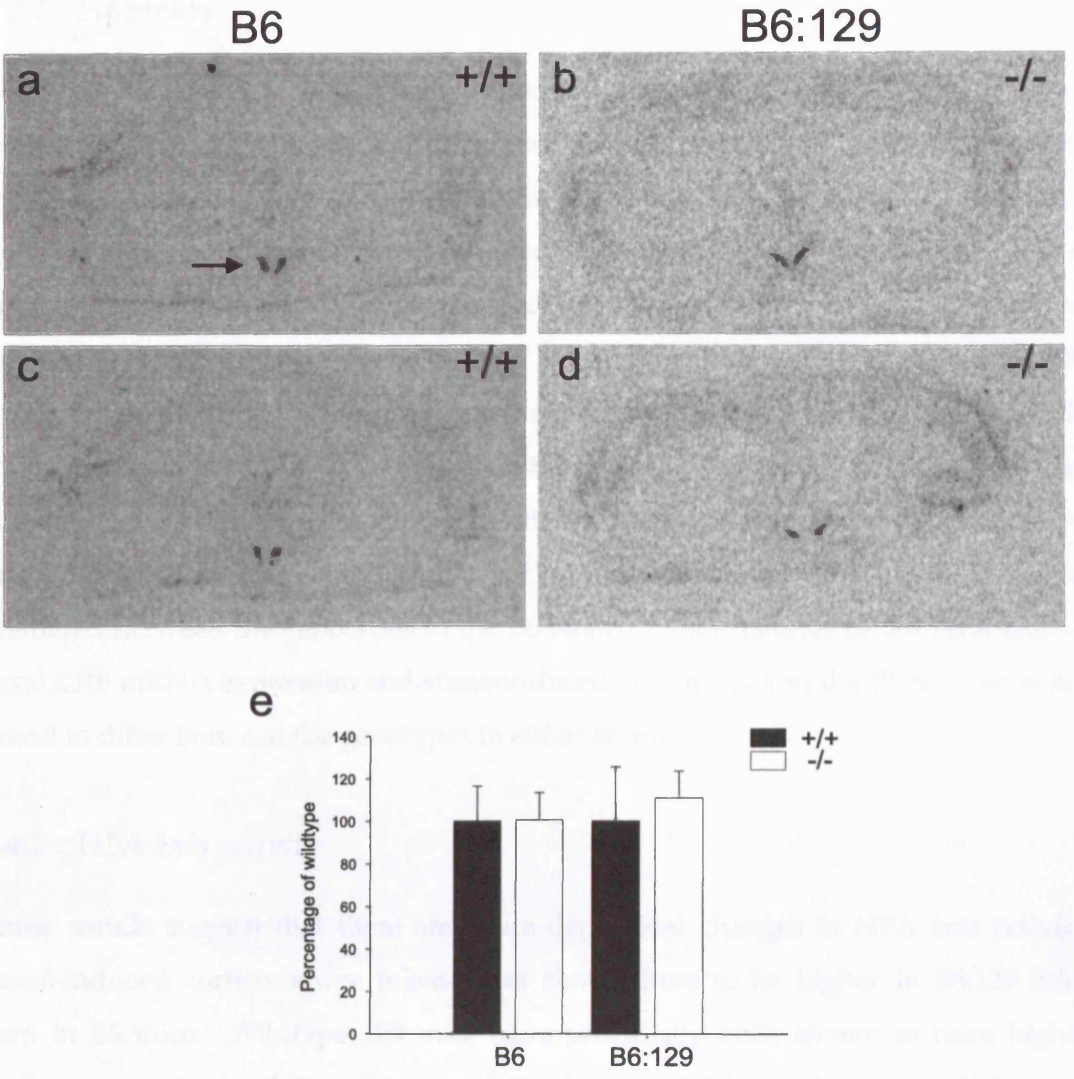

Figure 5.6: Restraint-induced cFos mRNA in the PVN (indicated by arrow in a) in B6 and B6:129 mice. Photographs show representative examples of brain sections labelled with ^{35}S-cFos in wildtype and NK1$^{-/-}$ mice on the B6 (a,c) and the B6:129 (b,d) backgrounds. Data are plotted as mean ± SEM (e). N=3-4.

5.4 Discussion

5.4.1 Summary

The experiments in this chapter were performed to see if knockout of the NK1 receptor altered HPA axis activity and if this differed between the strains. Increased release of corticosterone in response to restraint stress was found in the B6:129 strain compared to B6 although no difference was found between genotypes. Neurogenesis, which is known to be affected by corticosterone levels, was increased in the $NK1^{-/-}$ mouse on the B6:129 background compared to their wildtype counterparts. This corresponds with previously published work using the MF1 strain (Morcuende et al., 2003). In contrast, knockout of the NK1 receptor on the B6 strain had no effect on neurogenesis. Similarly, levels of hippocampal glucocorticoid receptors were found to be increased in the $NK1^{-/-}$ mouse on the B6:129 background compared to wildtype but were unaltered between the genotypes in the B6 strain. Other features of the HPA axis — basal CRF mRNA expression and stress-induced cFos mRNA in the PVN — were not found to differ between the genotypes in either strain.

5.4.2 HPA axis activity

These results suggest that there are strain-dependent changes in HPA axis activity. Stress-induced corticosterone release was shown here to be higher in B6:129 mice than in B6 mice. Wildtype 129 mice have previously been shown to have higher corticosterone than wildtype B6 mice (Peinado et al., 2005). Although no differences in basal corticosterone levels between B6 and the mixed B6:129 mice were found in the experiments presented here — either due to methodological differences or a strain-dose effect in the hybrid mouse — it seems likely that there are differences in HPA axis regulation between the strains. 129 mice have been shown to have an enlarged intermediate lobe of the pituitary compared to B6 mice (Kelly et al., 1997). Other work has shown that glucose metabolism also differs between 129 and B6 mice (Browne et al.,

1999). The higher corticosterone levels reported in the B6:129 mice here suggest that other aspects of the HPA axis may also differ between the B6:129 and the B6 strains. Unfortunately, the design of the experiments in this chapter to examine CRF and cFos mRNAs and GR expression, precluded comparison across strains. Future work to quantify levels of these molecules using immunoblot and real-time PCR will be of use in determining the basis of the increased corticosterone levels.

Despite these strain differences, there was no difference between the genotypes in corticosterone levels implying that the NK1 receptor is not an important factor in either inducing or terminating corticosterone release in response to stress, at least under the conditions tested here. Similarly, levels of basal CRF and stress-induced cFos mRNA did not appear to be genotype dependent. However, one component of the HPA axis, hippocampal GR, did show a genotype-dependent increase in expression levels in the B6:129 strain. GR is the low affinity receptor for corticosterone and is associated with termination of the stress response via a negative feedback mechanism (Korte, 2001). Increased GR expression should therefore lead to a reduced corticosterone response to a stressor and a quicker return to baseline levels. This was not found in the experiments here in which no genotype-dependent effects in corticosterone release were observed. An explanation for this may involve the severity of the stressor (30 minutes restraint) which may have masked any differences in the ability of the knockout on the B6:129 background to regulate its corticosterone levels differently than the wildtype. Another possibility is that different time points, either earlier or later than the ones chosen, may have uncovered differences between the genotypes in this strain. As well as acting to reduce corticosterone release, GR can also directly affect nuclear transcription (Hayashi et al., 2004; Herbert et al., 2006). When activated by corticosterone, GR can translocate to the nucleus and bind to specific response elements in the promoter regions of many genes. Additionally, GR also interacts with a wide range of transcription factors often resulting in histone modifications and chromatin remodelling (Hayashi et al., 2004). Furthermore, glucocorticoids may also act non-genomically through G-protein coupled membrane receptors or via the GR/hsp90 complex to have more rapid effects

on intracellular signalling (Croxtall et al., 2002).

Mineralocorticoid receptors (MRs) can also have a variety of effects both genomically and non-genomically in the brain and are likely to be affected by changes in the HPA axis between strains and genotypes. MRs show a more restricted expression pattern but levels are still high in key parts of the limbic circuitry such as the amygdala and the hippocampus (Korte, 2001; Herbert et al., 2006). MRs are activated by low levels of glucocorticoids, due to their high affinity, and maintain excitability of the HPA system. This is in opposition to GRs which act to reduce HPA axis activity and restore homeostasis after a transient rise in corticosterone. Clearly, a reduction in GR, as may be seen in the $NK1^{-/-}$ mouse, is likely to disrupt the normal function and control of the HPA axis. Although an increase in GR expression has been shown here in the $NK1^{-/-}$ mouse, expression levels of MR have not been studied and would provide useful information on their role in mediating the phenotype observed in these mice.

5.4.3 The HPA axis and affective disorders

As discussed in the Introduction in section 1.4.3, dysfunction of the HPA axis has been associated with the pathophysiology of depression and addictive disorders (Modell et al., 1997; Holsboer, 2000; Sarnyai et al., 2001; Goeders, 2002; Gillespie and Nemeroff, 2005). Mice generated with mutations in certain components of HPA axis have been postulated as animal models of affective disorders (Muller and Holsboer, 2006). Additionally, animal models have been developed in which early life stressors result in HPA axis dysfunction in adulthood and lead to depressive-like systems (Plotsky and Meaney, 1993). These will be described below.

5.4.4 Mice with mutations in HPA axis function

Mice overexpressing CRF show anxiogenic behaviour (Stenzel-Poore et al., 1994) and, concordantly, mice with either global or brain-specific knockout of the CRF1 receptor display reduced anxiety-related behaviour (Smith et al., 1998; Muller et al., 2003).

Knockout of GR has generally been shown to correlate with increased depression-related behaviours (Ridder et al., 2005; Boyle et al., 2006) whilst overexpression reduces these behaviours, especially in tests involving a stressor (Ridder et al., 2005). However, in some studies reduced anxiety-related behaviour has been found as a consequence of GR knockout (Tronche et al., 1999) and overexpression of GR has been shown to produce anxiogenic behaviour (Wei et al., 2004). In many of these studies behavioural tests have been confounded by profound alterations in HPA axis regulation, which has been suggested as a reason for some of these discrepancies (Muller and Holsboer, 2006).

$NK1^{-/-}$ mice on the B6:129 background have been shown here to overexpress hippocampal GR in comparison to wildtype counterparts. On different backgrounds, here and elsewhere (Rupniak et al., 2001; Santarelli et al., 2001; Fisher, 2005; Herpfer et al., 2005), $NK1^{-/-}$ mice have also been shown to display an anxiolytic and antidepressant-like phenotype. The GR overexpressing mice used by Ridder et al. (2005), as well as showing a resistance to developing helplessness, also show a significant increase in hippocampal BDNF, a feature that has also been found in $NK1^{-/-}$ mice (Morcuende et al., 2003).

In contradiction to this work, Wei et al. (2004) found that GR overexpression increased anxiety-related behaviour. They have explained this unexpected finding by arguing that these mice represent a model for increased emotional lability. In support of this hypothesis they also present work showing increased sensitisation to a rewarding stimulus, cocaine, and increased sensitivity to antidepressants. These GR overexpressing mice were also found to have increased CRF expression in the central nucleus of the amygdala and increased noradrenaline, serotonin and dopamine transporter expression in the locus coeruleus, dorsal raphe and ventral tegmental area, respectively. Additionally, as with the $NK1^{-/-}$ mouse here, they showed no differences in circulating corticosterone basally or after a stressor.

Differences between the work of Ridder et al. (2005), Wei et al. (2004) and that conducted with the $NK1^{-/-}$ mouse, indicate that GR is capable of modulating behaviour in a number of ways. Reasons for the discrepancies between each study

are numerous. Differences in distribution of GR and regulation of the HPA axis, the manner of testing and the tests used, and the genetic background of the mice studied, are all likely to have been of importance. Ridder et al. (2005) and Wei et al. (2004) each used different methods to overexpress GR. The former study generated mice carrying two additional copies of the GR using a yeast artificial chromosome. A twofold increase in GR protein was detected in the hippocampus although other work using this system has shown mRNA for GR to increase by between 20 – 60% in other brain and peripheral tissues, including the pituitary (Reichardt et al., 2000). In contrast, Wei et al. (2004) used a different transgenic construct driven by the calcium-calmodulin-dependent protein kinase II α promoter. They found the distribution of the transgene-specific GR to be primarily forebrain-specific with no expression in peripheral tissues. The highest expression levels were found in the hippocampus. The work presented in this chapter is the first attempt to examine levels of GR in NK1$^{-/-}$ mice. Here, we find an overexpression of GR in the hippocampus, specifically the CA1 region. As of yet, GR has not been compared in other hippocampal subfields, other brain regions, or peripheral tissue. Determination of these patterns will be of help in understanding the link between disruption of the NK1 receptor and changes in GR expression.

GR acts to terminate the corticosterone response to a stressor in many of the brain regions in which it is expressed, especially the hippocampus, the hypothalamus and in the pituitary (Korte, 2001). However, as detailed above, it can also have a variety of other effects within the cell, particularly the modulation of gene transcription (Hayashi et al., 2004).

As discussed extensively in this thesis, genetic background can have profound effects on behaviour, especially in anxiety-related models, and is also a major factor in determining an animals' HPA axis regulation. As such genetic background and its interaction with the HPA axis may have had a confounding effect on many of the behaviours examined in these studies (Muller and Holsboer, 2006).

More experiments are required to explain both the reasons for increased GR expression observed in the NK1$^{-/-}$ mouse and its significance. In addition, experiments

to determine differences in MR levels between strains and genotypes would also be instructive.

5.4.5 Early life stress and the HPA axis

Early-life experience is known to bring about enduring changes in the HPA axis. Adverse experiences during the perinatal period in particular are known to lead to later dysfunctions in HPA axis regulation and a depressive-like phenotype (Plotsky and Meaney, 1993). There is an extensive body of literature in this field detailing work using rodents and primates in different paradigms including maternal separation, handling and immune response. Procedures such as these lead to profound changes in neurobiology and behaviour that last into adulthood. For example, maternal separation in pups produces changes in stress-responsivity and feedback sensitivity of the HPA axis when tested as adults (Plotsky and Meaney, 1993; Heim et al., 2004). Additionally, marked behavioural changes also develop including anxiety-like behaviour, anhedonia, sleep disruption, decreased appetite and cognitive impairment (Huot et al., 2001; Heim et al., 2004). Adult neurogenesis is also reduced following early life stress (Korte et al., 2005).

Recently, work by Michael Meaney and colleagues has examined natural variation in maternal behaviour in rats and showed that this has an effect on the phenotype of these animals in adulthood (Liu et al., 1997; Francis et al., 1999; Weaver et al., 2004). Rats were divided into groups of high and low responders on the basis of a particular form of nursing provided by the dams. Offspring of low responders developed with hyperactive responses to stress and dysregulation of the HPA axis. This was found to correlate with low expression levels of GR and the expression of a behavioural phenotype including anxiety-like behaviour and anhedonia (Liu et al., 1997). These pups were observed to grow up as low responding mothers and, vice versa, the offspring of high responders became high responding mothers. Interestingly, it was also found that by cross-fostering pups this trend could be reversed implying

an epigenetic mechanism (Francis et al., 1999). Examination of chromatin structure in these animals revealed that differences in DNA methylation and histone acetylation existed between the groups, in particular, in the region of the GR promoter (Weaver et al., 2004). As such, in high responders transcription factors could more easily bind this promoter leading to increased expression of GR.

Further studies have shown a number of different signalling systems and behaviours are also affected by these differences in maternal care including GABAergic pathways (Caldji et al., 2003), long-term potentiation and spatial learning (Liu et al., 2000), and survival of new-born cells in the hippocampus (Bredy, Grant, Champagne and Meaney, 2003). Additionally, although these changes are relatively stable, interventions such as environmental enrichment (Francis et al., 2002; Bredy, Humpartzoomian, Cain and Meaney, 2003) or treatment with agents to affect the chromatin structure (Weaver et al., 2005, 2006) are able reverse this programming.

In this thesis, differences between HPA axis activity, anxiety-related behaviour and morphine-induced locomotion have been seen between the strains. These observations may in part be explained by differences in maternal behaviour and/or DNA methylation between the strains. B6 mice have been compared to BALB/c mice in many studies examining genotype-phenotype interactions due to their strikingly different behavioural profiles. B6 mice show low anxiety in novel environments whilst BALB/c mice are substantially more neophobic (Belzung and Griebel, 2001). In studies to examine maternal behaviours, B6 mice have been shown to have upregulated $GABA_A$ receptor binding in the amygdala compared with BALB/c mice, an effect that was, however, abolished after crossfostering to BALB/c mothers (Caldji et al., 2004). The effect of mothering behaviour has not been examined in any of the 129 substrains but their anxiety-like profile and hypolocomotor response to novel environments suggests that they exhibit a behavioural profile that has more in common with the BALB/c than the B6 strain. This in turn would imply that similar differences may exist in mothering behaviour between the 129 and B6 mice as have been found between BALB/c and B6 mice by Caldji et al. (2004).

Furthermore, as the NK1$^{-/-}$ mice used in this study were bred on homozygous backgrounds, an intriguing possibility is that there may be variations in maternal care between the genotypes that lead to their differential expression of GR. Although much of the work with antagonists would suggest that disruption of the NK1 signalling pathway is able to have an acute effect on the behaviours we have examined, there is still a possibility that the effect of mothering behaviour may be important for the experiments presented here. Changes in GR expression in the work by Meaney have been associated with a number of other alterations in signalling that have also been found in NK1$^{-/-}$ mice, including differences in the monoaminergic system (Santarelli et al., 2001; Guiard et al., 2004; Fisher, 2005; Herpfer et al., 2005) and hippocampal BDNF (Morcuende et al., 2003). One interpretation of these findings is that an NK1 receptor-dependent pathway is recruited in BALB/c mice in the study by Caldji et al. (2004) and in wildtype B6:129 in this study leading to a reduction of GR expression. Differences in the genotypes observed between the NK1$^{-/-}$ and wildtype mice here are a result of removal of this pathway. In contrast, B6 mice do not recruit this pathway, possibly due to their lower levels of stress, and consequently have a high level of GR expression regardless of whether they have a functional NK1 receptor or not.

5.4.6 Neurogenesis

A related finding is that NK1$^{-/-}$ mice on the B6:129 strain were shown here to exhibit increased hippocampal neurogenesis as previously published in the MF1 and 129:B6 strains of NK1$^{-/-}$ mice. This may be related to their increased expression of GR as these receptors are known to modulate neurogenesis, possibly via their regulation of HPA axis activity (Duman and Monteggia, 2006). Prolonged elevated corticosterone levels have been shown to decrease neurogenesis and the survival of newly born progenitor cells (Cameron and Gould, 1994; Gould and Tanapat, 1999; Wong and Herbert, 2004; Lledo et al., 2006). The finding that neurogenesis is increased in NK1$^{-/-}$ mice on the B6:129 background is likely to have many implications for the behavioural

phenotype of these mice as neurogenesis has been postulated to have a role in several behaviours involving the hippocampus.

5.4.6.1 *Neurogenesis in learning and memory*

Neurogenesis has been shown to be important for learning and memory processes. As such, hippocampal-dependent learning increases neurogenesis (Gould et al., 1999) and blocking neurogenesis results in impairments in hippocampal-based tasks (Shors et al., 2001; Snyder et al., 2005). The results in this chapter would therefore predict an enhancement of hippocampal-dependent learning in the $NK1^{-/-}$ mouse on the mixed B6:129 background. However, in a comprehensive study of learning and memory behaviours in the $NK1^{-/-}$ mouse on the mixed MF1 background few differences were found between the genotypes in any of the tests (Gadd, 2003). Both hippocampal tasks and non-hippocampal tasks were examined and included the Morris water maze, fear conditioning, working memory tasks and Rota-Rod performance. A subtle increase in selectivity during probe trial behaviour in the water maze was detected in $NK1^{-/-}$ mice, suggesting they may be slightly quicker at demonstrating spatial memory. However, this is a minor difference in comparison to the increases in neurogenesis observed. More recent work examining the role of neurogenesis in spatial memory has shown that preventing neurogenesis impairs long- but not short-term performance in the Morris water maze (Snyder et al., 2005). New hippocampal neurons in the dentate gyrus may then be required for the formation and/or consolidation of long-term memories and the encoding of time (Aimone et al., 2006). Further investigation of the $NK1^{-/-}$ mouse using different paradigms and experimental conditions may uncover more interesting behavioural differences between the genotypes.

5.4.6.2 *Neurogenesis and antidepressants*

As well as learning and memory, neurogenesis is also believed to be somewhat involved in the pathophysiology of depression and the actions of antidepressants

(Malberg et al., 2000; Duman et al., 2001; Santarelli et al., 2003). It has been more difficult to determine the precise role of neurogenesis in depression mainly due to the difficulties in reliably assaying traits related to depression in animal models (Cryan and Mombereau, 2004). This has led to inferences about the role of newly-born granule cells in the disease based on the effects of established antidepressants on neurogenesis and in behavioural models. Clinically, depression has been linked to reduced hippocampal volume (Sheline et al., 1996) and antidepressant treatment is believed to ameliorate this reduction (Czeh et al., 2001) adding support to the neurogenesis hypothesis of depression. However, questions still remain as to whether these findings are artifacts linked to treatment of the disease or other social conditions such as drug abuse or poverty, which are also associated with depression. Despite these caveats it seems likely that the increases in neurogenesis observed here in the $NK1^{-/-}$ mouse on the B6:129 background — and previously on the mixed MF1 background (Morcuende et al., 2003) — are linked to the antidepressant phenotype observed in the knockout mouse and in wildtype mice after administration of NK1 receptor antagonists. Although the $NK1^{-/-}$ mouse has been studied using the forced swim and the tail suspension tests (Rupniak et al., 2001), further examination using paradigms involving a longer temporal component such as repeated psychosocial stress may help to elucidate the contribution of newly-born neurons and the role of the NK1 receptor in depression. An attempt to link the observed increase in GR expression in the $NK1^{-/-}$ mouse on the B6:129 background with the increase in cell proliferation would be a promising direction for future study. If these two features were linked, chronic antagonism of the GR receptor would be expected to decrease neurogenesis and have consequent effects on behaviour.

5.4.7 Strain differences in neurogenesis and the HPA axis

NK1 receptor knockout did not affect either GR expression or neurogenesis in the B6 strain whereas there was a large effect in the B6:129 strain. As the B6:129 strain

were also shown to have elevated levels of corticosterone after a stressor compared to B6 it could be hypothesised that a HPA axis-mediated pathway involving the NK1 receptor was recruited in the B6:129 mice but not in the B6 mice. One site that may be important in mediating this response is the locus coeruleus (LC). The LC is activated by numerous stressful stimuli as shown by patterns of cFos expression after stress (Ceccatelli et al., 1989; Passerin et al., 2000), the electrophysiological response of its neurons (Abercrombie and Jacobs, 1987) and by release of noradrenaline into regions that it innervates (Gresch et al., 1994; Pacak et al., 1995; Quirarte et al., 1998). The NK1 receptor is expressed in very high levels in the LC (Maeno et al., 1993; Chen et al., 2000) and NK1 antagonists (Millan et al., 2001; Maubach et al., 2002) and substance P (Guyenet and Aghajanian, 1977; Cheeseman et al., 1983) both affect the firing properties of its neurons. $NK1^{-/-}$ mice have been shown to have increased cortical noradrenaline under anaesthesia, an effect that is mimicked by NK1 receptor antagonists (Fisher, 2005). Increased serotonin efflux after SSRI administration has also been shown in the $NK1^{-/-}$ mouse (Froger et al., 2001) and this is believed to be dependent on increases in the noradrenergic system (Gobbi et al., 2006). Importantly, the autoregulatory receptors for noradrenaline and serotonin, the 5-HT_{1A} receptor and the α2-adrenoceptor, respectively, are believed to be involved in these changes through a reduction in their functional activity (Froger et al., 2001; Santarelli et al., 2001, 2002; Fisher, 2005). Increased activation of the LC in response to a stressor then may be altered in animals lacking a functional NK1 receptor. The larger this activation the more important the presence of NK1 receptors would be supposed to be. The larger increase in HPA axis activity observed in B6:129 mice compared with B6 mice may mean that a pathway involving the LC is more strongly stimulated in this strain and hence the role of the NK1 receptor is also of more importance.

The behavioural experiments presented in this thesis also support a role for the LC in mediating some of the NK1 receptor-dependent behaviours. Alterations in the opiate system seen in some $NK1^{-/-}$ mice may be involve the LC as the LC has also been implicated in the development of opiate dependence (Nestler et al., 1994; Nestler

and Aghajanian, 1997; Nestler et al., 1999) and both μ opioid and NK1 receptors are localised on these cells' somata (Chen et al., 2000; Moyse et al., 1997).

The expression of other tachykinin receptors could also be involved in the observed strain differences. Microdialysis studies have implicated NK3 receptor activation in the LC as being essential for the effects of NK1 receptor agonists in this region (Bert et al., 2002). Developmental compensations after genetic disruption of the receptor could show interstrain variability and account in part for the observed differences.

5.4.8 Interactions between strain and genotype

There are several other studies in which similar strain-dependent effects on phenotype have been found in genetically-modified mice involving the HPA axis. Peinado et al. (2005) used two null mouse models to study the role of prohormone convertase (PC2) and its helper protein 7B2. By breeding the mutations into both B6 and 129 backgrounds they were able to study their affects on HPA axis activity. Wildtype B6 mice were found to have lower corticosterone levels than wildtype 129 mice, a result partially backed up by findings in this thesis. Additionally B6 mice also had higher blood glucose levels. The 7B2 mutation results in a lethal phenotype in the 129 background due to the development of a Cushing's-like disease. In the B6 mutant, however, mice with the null mutation survived and showed decreased corticosterone and increased blood glucose levels, most likely due to their increased adrenal resistance to ACTH stimulation. The authors also show that the B6 background strongly predominates over the 129 background as a single generational cross into the B6 background results in protection from the lethal effects of the 7B2 mutation (Peinado et al., 2005).

In a study examining the effect of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) knockout in the mouse, similar strain-genotype interactions have been found. 11β-HSD1 is an enzyme responsible for regulating aspects of HPA axis activity via the conversion of inactive 11-dehydroxycorticosterone (11-DHC) into

active glucocorticoids. It has been associated with many disease states including metabolic disturbances and psychiatric conditions including depression and PTSD (Carter, 2006). On the original mixed 129/MF1 background the 11β-HSD1 knockout showed decreased glucocorticoid negative feedback as well as a shift in peak circadian levels and elevated a.m. levels of plasma corticosterone (Kotelevtsev et al., 1997; Harris et al., 2001). After backcrossing onto a congenic B6 background although some of the metabolic and cognitive effects seen in the MF1/129 remain, basal corticosterone and ACTH levels no longer differ between genotypes. Additionally, the impaired negative feedback, as assayed by the return to baseline levels of corticosterone and ACTH after restraint stress, is no longer present in the 11β-HSD1 knockout on the B6 background. Comparison of the mutation on a number of backgrounds led the authors to suggest that genetic modifiers within the 129 strain contribute to the attenuation of negative feedback seen in the 11β-HSD1 knockout. Further work examining molecular components of the HPA axis in these mice, in particular the glucocorticoid receptor (GR), suggests that a key difference between the strains may involve regulation of GR (Carter, 2006).

Interestingly, the gene for GR uses several different promoters and can be regulated by many different transcription factors. Among the transcription factors known to regulate expression of GR is zif268 which binds to a promoter region in the non-coding exon 1 region of the GR gene (Weaver et al., 2004). Additionally, glucocorticoids are known to exert many of their effects via a pathway involving zif268 (Revest et al., 2005). zif268 has been implicated in many behaviours including drug addiction (Lee et al., 2005; Valjent et al., 2006), learning and memory (Davis et al., 2003) and pain processing (Wong, 2006).

These studies show that genetic background interacts with the HPA axis in a number of ways. Altering a component of this finely-tuned system can result in changes in many of its other parts as it tries to return to homeostasis.

5.4.9 Summary and conclusions

In this chapter a number of aspects of HPA axis regulation and a related phenomenon, neurogenesis, have been examined in two strains of $NK1^{-/-}$ mouse. Changes across strain and genotype indicate that there are important interactions between the NK1 receptor and stress-responsive systems. The importance of genetic background has again been highlighted and show that care must be taken when interpreting the results of studies using mice, especially when genome engineering and behavioural tests are used. In the final chapter, findings from this thesis will be brought together to consider the implications of this work and possible areas of future study.

Chapter 6

General Discussion

6.1 *Introduction*

Genetic background has a profound effect on mouse phenotype. The experiments presented in this thesis demonstrate how the effect of a genetic manipulation can be drastically altered by the strain of mouse it is examined in. Specifically, the aim was to study the physiological role of the NK1 receptor. Previous research indicated that this receptor may be a promising therapeutic target for the treatment of opiate addiction or affective disorders such as depression or anxiety. A mouse line lacking a functional NK1 receptor was created using homologous recombination. Pharmacological study using antagonists had been limited by species differences in the primary structure of the receptor protein. The NK1 knockout mouse presented an interesting phenotype including anxiolysis, antidepressant-like behaviour and a reduced potential for opiate addiction. For reasons of experimental rigour, and to enable the continued study of more complex cognitive behaviours, the receptor mutation was transferred onto a different genetic background. The experiments in this thesis have examined the effect of NK1 receptor disruption in three different background strains.

6.2 *Summary of results*

The experiments in the first chapter compare the effect of NK1 receptor knockout in the C57BL/6 strain (B6) with its effect in the existing MF1:129:B6 mixed background (MF1).

The attenuation of morphine-induced hyperlocomotion observed in $NK1^{-/-}$ mice on the MF1 background was lost in the B6 background. Additionally, the increased basal locomotion seen in $NK1^{-/-}$ mice on the MF1 background also disappeared in the B6 strain.

In the light/dark exploration box (LDEB) several behaviours associated with anxiolysis that were increased in the $NK1^{-/-}$ mouse on the MF1 strain showed no difference between genotypes in the B6 background. The one behaviour that did differ between genotypes in the B6 strain was number of transitions between compartments, a behaviour normally associated with exploratory activity. $NK1^{-/-}$ mice performed less transitions than wildtype indicating perhaps a decrease in exploration and an anxiogenic effect of the mutation.

The next set of experiments involved transferring the mutation onto a mixed B6:129 background. It was hoped that this would recapitulate, at least in part, the phenotype seen in the mixed MF1 background. The B6:129 mouse had a lower hyperlocomotor response to morphine than B6 mice even at the highest dose. Surprisingly, at one dose a strong stimulating effect on locomotion was observed in the $NK1^{-/-}$ mouse that was not seen in their wildtype counterparts.

In the LDEB no significant differences were found between the genotypes although the general phenotype was one that was intermediate between the MF1:129 and B6 strains. There were, however, trends towards an anxiolytic phenotype in the knockout.

Finally, a set of experiments was undertaken to examine aspects of the HPA axis in the B6 and B6:129 strains. Stress-induced corticosterone levels were shown to be higher in the B6:129 strain than the B6 strain. No differences in corticosterone levels were attributable to genotype. Hippocampal neurogenesis may be an important part of the pathophysiology of depression or of antidepressant treatment. It was previously shown to be increased in the $NK1^{-/-}$ mouse on the MF1 background. Here, it was shown not to differ between genotypes on the B6 background. Creation of the $NK1^{-/-}$ mutation on the mixed B6:129 background recapitulated the increase in neurogenesis previously observed in the knockout. Levels of glucocorticoid receptor (GR) were also

increased in the NK1$^{-/-}$ mouse compared to wildtype in the B6:129 strain. In contrast, no difference between genotypes in neurogenesis or GR expression were seen in the B6 strain. Basal corticotrophin releasing factor (CRF) and stress-induced cFos mRNAs in the PVN were found not to differ between genotypes on either strain.

The results presented have shown a key interaction between strain and genotype in the NK1$^{-/-}$ mouse. Of central importance seems to be regulation of systems mediating anxiety-like behaviours in the mouse and their genetic component. The HPA axis contributes to the expression of these behaviours and shows a high degree of variability between animals, partly as a result of genetic background but additionally due to environmental conditions. Morphine acts in a number of ways on the nervous system and how these effects at a molecular, cellular and systems level are translated into behaviour is a complex issue demanding further study. This will enable more successful treatment of those unfortunate enough to suffer from opiate addictions and and will also aid in the medical use of opiates to treat conditions such as chronic pain.

Taken together, the results in this thesis demonstrate the profound differences that exist between different strains of mice. Furthermore, these experiments show that a specific mutation can have a variety of phenotypic effects which are strain-dependent. Clearly there is an important interaction between strain and genotype which can lead to surprising results. Given what we know about the underlying biological complexity this should not come as a surprise. One of the key criticisms, or at least caveats, of studies using knockout animals is that developmental compensations will occur. Physiological systems are comprised of many finely-tuned components and the removal of one protein or signalling molecule will often trigger a host of changes designed to return the network to homeostasis. Careful consideration should be applied when conducting or interpreting experiments of this nature.

6.3 *Future directions*

Although the work presented in this thesis has shown that there are complicated interactions between strain and genotype, particularly with respect to the $NK1^{-/-}$ mouse, there exist many opportunities for future study. The key finding is that genetic background has a major effect on morphine-induced locomotion, behaviour in the light/dark exploration box, components of the HPA axis, and neurogenesis. Importantly, these differences in genetic background drastically altered the effect of NK1 receptor disruption. Throughout this thesis I have tried to present possible explanations for these findings and suggest areas of future study. Here, I will review some of the common themes that are applicable to all of the findings.

6.3.1 *Receptor distribution and activity*

Firstly, it seems clear that to properly understand the function of substance P and the NK1 receptor in the different strains, a comprehensive study of their relative distribution and activity in the central nervous system is required. In behavioural studies heterozygotes seem to resemble $NK1^{-/-}$ mice indicating a haplosufficiency of the NK1 gene (Santarelli et al., 2001). However, differences other than absolute expression level could also contribute to altering the function of the NK1 receptor between different genetic backgrounds. Changes in the distribution of the receptor or its functional activity could have profound effects on its involvement in different phenotypes. These may be conferred by changes in both the exonic or intronic sequences as has been noted with other receptors, leading to differential trafficking and/or functional activity between the strains (Zhou et al., 2001). Furthermore, this information is also required for other receptors, proteins, transmitters or other components that may be involved in the behaviours or features of a phenotype that show strain-genotype interactions. Of particular interest to the work in this thesis would be a comparison of μ opioid receptors and of glucocorticoid receptors between the strains.

6.3.2 *Other tachykinins*

The role of other tachykinins has been discussed in this thesis but almost completely ignored in studies using NK1$^{-/-}$ mice. Although dogma suggests that the other neurokinin receptors are unlikely to be involved in regulation of the same systems as the NK1 receptor, there has been little work to specifically examine NK2 and NK3 receptors in the NK1$^{-/-}$ mouse. Likewise, the other tachykinin agonists, especially NKA, may have a role to play in modulating NK1 receptor-mediated circuits along with substance P (Maggi and Schwartz, 1997). Evidence is accumulating that NK2 and NK3 receptors (or their ligands) may be involved in similar physiological actions as substance P and the NK1 receptor (Culman and Unger, 1995; Piot et al., 1995; Teixeira et al., 1996; Salome et al., 2006) and this is an area that therefore warrants further study.

6.3.3 *Epigenetic effects*

The important and highly complex manner in which genes and environment interact is now an avidly studied area of research. Despite this, these interactions are often not fully examined in work using genetically modified mice. This is of particular relevance due to the variety of breeding strategies which are implemented. Both the behaviour of parents and offspring can vary as the result of genetic manipulation. These changes although having a genetic origin may lead to epigenetic changes which may then be passed down non-genomically as shown by cross-fostering studies (Caldji et al., 2004). An interesting avenue of future study would be to examine the role of the NK1 receptor in these processes. NK1$^{-/-}$ mouse pups are known to emit fewer neonatal vocalisations suggesting the possibility that their adult phenotype may result from differential maternal responses (Rupniak et al., 2000). Similarly, changes in GR resulting from NK1 receptor disruption in dams may also give rise to differences in mothering behaviour with consequent effects on behaviour. Experiments using cross-fostering between litters and examining chromatin modifications in these mice may provide useful information about how the NK1 receptor is involved in regulation of

these systems and why there are such pronounced differences between the strains (Mager and Bartolomei, 2005).

6.3.4 *Regional-specific studies of the NK1 receptor*

More experiments are required to determine the precise site of action of substance P or the NK1 receptor in each of the phenotypes revealed. The work by Gadd et al. (2003) was useful in determining the amygdala as an important mediator of effects on anxiety and morphine-related behaviours. However, although selective, the substance P-saporin conjugate still results in the destruction of many cells which may be crucial for normal function and so a method of deleting the receptor in a region-specific manner whilst leaving the cell population intact would be preferable (Ryding et al., 2001). Several methods now exist such as the introduction of antisense oligonucleotides or the conditional knockout of a receptor using site-specific mutagenesis such as the Cre-LoxP system (Morozov et al., 2003; Glaser et al., 2005). Additionally, restoration of function by using viral reintroduction of the NK1 gene in $NK1^{-/-}$ mice would also give useful information about the specific brain areas which are important in producing the phenotype seen in $NK1^{-/-}$ mice. Furthermore, using a temporally restricted knockout strategy to ameliorate any developmental compensations that may occur with the $NK1^{-/-}$ mouse used in this study, is also an option (Morozov et al., 2003).

6.4 *Final conclusions*

The work presented in this thesis raises important issues that are relevant to all studies using genetically modified mice. That background strain can have such a profound influence on the effect of a genetic manipulation is a finding that warrants much future study and consideration. Many genetically modified animals have been proposed as models for various affective disorders. Targeted mutations allowing the study of changes in a single gene provide an attractive way of examining many biological

processes. However, epistatic interactions with the background that the mutation is expressed on, the influence of linked genes, and developmental compensations can often confound these findings. Different mouse strains provide a rich source of behavioural phenotypes and may, even without genetic manipulation, serve as animal models of disease. As such, comparison of strains which differ markedly might provide useful information on the underlying physiology that contributes to behavioural differences. Using animals exhibiting a trait, such as increased anxiety-like behaviour, as an enduring feature of the particular strain, rather than because of the disruption of a single gene, can overcome some of these problems. Complex traits are likely to involve multiple genetic and environmental factors and may be more rigourously studied in this manner.

Vulnerability to external events, such as stress, as a factor involved in the development of affective disorders or drug addiction is well documented in human patients and is likely to be in part conferred by genetic background. It is likely that a similar spectrum of vulnerability also exists between different strains of mice. Healthy humans are unaffected by antidepressant treatment. Additionally, many human patients do not respond to treatments that are effective in other patients. These phenomena may be investigated by exploring the various vulnerabilities or protective factors implicated by the different responses observed among mouse strains.

Vast amounts of time and money are currently spent developing new treatments for disease. Drug addiction and depression/anxiety disorders are two of the most costly burdens on world health. This is at a personal, financial and societal level. Many drugs show efficacy in only a small percentage of the patient population and this often leads to lengthy and complex changes to their treatment regimen before finding an appropriate program. This is especially the case with depression as our diagnostic tools and disease classifications are still relatively crude. The full sequencing of the human genome is a huge leap forward in science and should in time provide an aid to our understanding of the genetic component underlying disease. Similarly, it may become possible to begin tailoring treatments to specific patients on the basis of their individual

genetic profile. The variety of mouse strains available for laboratory research is a valuable resource that needs to be fully utilised by the scientific community, especially those using mutant models. Careful experimentation and analysis using these mice will provide important information on the genetic bases of complex diseases.

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Appendix A

Detailed protocols

A.1 Laboratory solutions

Phosphate buffer (PB; 0.1 M; pH 7.4):

190 mM	NaH_2PO_4	(BDH, Poole, UK)
810 mM	Na_2HPO_4	(BDH)

Tris buffer (0.15 M; pH 7.6):

127 mM	Tris HCl	(Sigma, Poole, UK)
23 mM	Tris base	(Sigma)

Heparanised phosphate-buffered saline (PBS):

0.9 %	NaCl	(Baxter, Lessines, Belgium)
5 AU/ml	heparin	(CP Pharmaceuticals, Wrexham, UK)
5 mM	PB	

Normal Goat Serum (NGS) solution:

0.1 M	PB	
3 %	NGS	(Vector Laboratories, Burlingame, USA)
0.3 %	Triton X-100	(BDH)
0.02 %	NaN_3	(Sigma)

A.2 Genotyping

All mice used in this thesis were checked for genotype using polymerase chain reaction (PCR) of tail tip DNA. The protocols for DNA extraction and the NK1 PCR reaction are given over the next few pages. Figure 2.3 in the Materials and Methods shows an example gel.

A.2.1 DNA extraction from tail tip

- Remove 5 mm of tail tip using a razor blade and place in a 1.5 ml microfuge tube.
- Add 750 μl tail lysis buffer[1] and 22.5 μl Proteinase K (20 mg/ml; Sigma) to tube and leave overnight at 55 °C.
- Vortex and spin for 3 min at 13,000 g.
- Transfer supernatant to fresh tubes leaving hair etc.
- Add 200 μl Protein Precipitation Solution (Puregene, Minneapolis, USA) and vortex for 20 s.
- Centrifuge for 3 min, 13,000 g. A tight light coloured pellet should form.
- Pour supernatant into new tubes containing 600 μl 100 % isopropanol and mix thoroughly by gentle inversion. A stringy white precipitate should form.
- Centrifuge for 3 min at 13,000 g.
- Discard supernatant and add 300 μl 70 % ethanol.
- Centrifuge for 3 min at 13,000 g.
- Remove supernatant and leave open to air dry for 1–2 h.
- Add 250 μl TE. Tap and quick spin again.
- Leave at 4 °C overnight to dissolve precipitate.

[1]Tail lysis buffer: 50 mM Tris pH 8, 100 mM EDTA pH 8, 10 mM NaCl, 1 % SDS; Sigma

A.2.2 NK1 PCR protocol

- Make up PCR reaction mix:

– dH_2O	32.50 μl
– *Taq* DNA polymerase 10 x buffer (Promega)	5.00 μl
– 25 mM $MgCl_2$ (Promega)	3.00 μl
– 10 μM dNTP mix (Promega)	1.00 μl
– NeoF[1] (0.5 μg/μl; Sigma Genosys, Cambridge, UK)	1.00 μl
– NK1-F[2] (0.5 μg/μl; Sigma Genosys)	1.00 μl
– NK1-R[3] (0.5 μg/μl; Sigma Genosys)	1.00 μl
– *Taq* DNA polymerase (5 u/μl; Promega)	0.50 μl
– DNA sample	5.00 μl
– **Total**	**50.00 μl**

- Place tubes in PCR temperature cycler (PTC-100 Programmable Thermal Controller, MJ Research, Boston, USA) and run the following programme repeating steps 2 to 4, 34 times:

1.	95 °C	5 min
2.	60 °C	30 s
3.	72 °C	30 s
4.	94 °C	30 s
5.	60 °C	30 s
6.	72 °C	5 min

[1]NeoF: 5′-GCAGCGATCGCCTTCTATC-3′
[2]NK1-F: 5′-CTGTGGACTCTAATCTCTTCC-3′
[3]NK1-R: 5′-ACAGCTGTCATGGAGTAGATAC-3′

- Add 5 μl loading buffer[4] and mix well.
- Run 11 μl of each sample on a 2 % agarose gel in TBE buffer (National Diagnostics) containing 10 μg/ml ethidium bromide (Sigma) at 120 mV for approximately 1 h.
- Visualise and photograph under ultraviolet transillumination.

[4]Loading buffer: 0.25 % bromophenol blue (Sigma), 0.25 % xylene cyanol FF (Sigma), 30 % glycerol (BDH)

A.3 Immunocytochemistry

Immunocytochemistry was used in this thesis to identify and quantify tissue constituents. The theory behind the technique is described in section 2.2 in the Materials & Methods chapter. Here, a detailed description of the protocols used for chromogenic and fluorescent detection methods is given.

For the BrdU staining it was necessary to pre-treat sections by incubating in 2 N HCl for 30 min followed by 0.1 M boric acid for 10 min.

A.3.1 Immunocytochemistry with chromogenic detection using DAB

Gentle agitation of sections on a tissue rocker at RT is used for all steps, except where indicated.

- Rinse sections in 0.1 M PB.
- Quench endogenous peroxidase activity by incubating sections in 0.6 % H_2O_2 in 0.1 M PB for 30 min.
- Block non-specific binding by incubating in Normal Goat Serum (NGS) solution for 1 h[1].
- Incubate sections in primary antibody in NGS solution overnight at RT or for 2–3 d at 4 °C.
- Wash sections 3 x 10 min in 0.1 M PB.
- Incubate sections in biotinylated anti-rabbit IgG raised in goat[2] (1:500 in NGS; Vector Laboratories) for 90 min.
- Prepare avidin-peroxidase solution using Vectastain Elite ABC Kit (Vector Laboratories) and leave on rocker for 30 min before using.

[1]The serum used must be that of the animal in which the secondary antibody was raised.

[2]The secondary antibody must be raised that against the animal in which the primary antibody was raised.

- Wash sections 3 x 10 min in 0.1 M PB.
- Incubate sections in avidin-peroxidase solution for 30 min.
- Wash sections 3 x 10 min in 0.1 M PB.
- Make up DAB solution[3] from kit.
- Incubate in DAB solution for 3–10 min.
- Quench in dH_2O and then transfer to 0.1 M PB.
- Mount sections onto gelatine-coated slides from 0.01 M PB and leave to air dry.
- Dehydrate sections through ethanol series (dH_2O, 70 % ethanol x 2, 95 % ethanol x 2, 100 % ethanol x 2; 2 min each).
- Clear in Histoclear (2 min x 2; National Diagnostics).
- Coverslip using DPX mounting medium (BDH).

[3]DAB solution: 2 drops buffer, 4 drops DAB, 2 drops peroxidase, 2 drops nickel in 5 ml dH_2O; Vector Laboratories

A.3.2 *Immunocytochemistry with fluorescent detection*

Gentle agitation of sections on a tissue rocker at RT is used for all steps, except where indicated.

- Rinse sections in 0.1 M PB.
- Block non-specific binding by incubating in Normal Goat Serum (NGS) solution for 1 h[1].
- Incubate sections in primary antibody in NGS solution overnight at RT or for 2–3 d at 4 °C.
- Wash sections 3 x 10 min in 0.1 M PB.

For directly-conjugated Alexa 488:

- Incubate sections in the dark in AlexaFluor488 (Molecular Probes, Oregon, USA) for 2 h.
- Wash sections 3 x 10 min in 0.1 M PB in the dark.
- Rinse sections in the dark in 0.01 M PB
- Mount sections onto gelatine-coated slides and leave to air dry in the dark.
- Coverslip with Citifluor (Citifluor, London, UK).

Or, for FITC fluorescence (avidin-biotin method):

- Incubate sections in biotinylated anti-rabbit IgG raised in goat[2] (1:200 in NGS solution; Vector Laboratories) for 90 min.
- Wash sections 3 x 10 min in 0.1 M PB.

[1]The serum used must be that of the animal in which the secondary antibody was raised.

[2]The secondary antibody must be raised that against the animal in which the primary antibody was raised.

- Incubate sections in the dark in Fluorescin Avidin D (1:200 in 0.1 M PB; Vector Laboratories) for 1 h.
- Wash sections 3 x 10 min in 0.1 M PB in the dark.
- Rinse sections in the dark in 0.01 M PB
- Mount sections onto gelatine-coated slides and leave to air dry in the dark.
- Coverslip with Citifluor (Citifluor).

A.4 Immunoblotting

As the antibody for the glucocorticoid receptor had not been used in our laboratory before, an immunoblot was conducted to ensure binding to an antigen of appropriate size and distribution was observed.

A.4.1 Sample preparation

- Remove brains and dissect on ice before freezing on dry-ice.
- Homogenize samples in 0.5–1 ml RIPA buffer containing a cocktail of proteinase and phospatase inhibitors[1].
- Incubate samples on ice for 2 h.
- Centrifuge samples for 15 min (12,000 g, 4 °C) and collect supernatant.
- Normalise samples for protein content using BCA kit (Pierce, Rockford, IL).

A.4.2 Running the gel

- Mix samples with 1 X loading buffer[2] and boil for 5–10 min to denature proteins.
- Load 10 μl (10 μg total protein) of each sample in 10 % SDS polyacrylamide gel (Bio-Rad).
- Run gel for 90 min at 100 V.
- Transfer proteins to Polyvinylidine difluoride (PVDF) membrane (Bio-Rad) at 4°C for 45 min at 100 V.

[1]RIPA buffer: 1 % NP40, 20 mM HEPES pH 7.4, 100 mM sodium chloride, 100 mM sodium fluoride, 1 mM sodium orthovanadate (Na_3Vo_4), 10 μg/ml leupeptin and 1 μg/ml; Sigma, Poole, UK

[2]Loading buffer: 10 % w/v sodium dodecyl sulphate; 50 % v/v glycerol and 0.5 % w/v bromphenol blue; Sigma

A.4.3 Detection of bands

- Block non-specific antibody binding with 5 % Bovine serum albumin (BSA) and PBS / 1 % Tween for 1 h at RT.
- Incubate with GR antibody at 1:1000 in 5 % Bovine serum albumin (BSA) and PBS / 1 % Tween overnight at 4°C
- Wash 6 x 5 min in PBS / 1 % Tween.
- Incubate with horseradish peroxidase-conjugated mouse anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK) at 1:2000 for 90 min at RT.
- Wash 6 x 5 min in PBS / 1 % Tween.
- Identify bands using the enhanced chemiluminescence (ECL) detection system and X-ray film for 1–20 min (Amersham Biosciences).

A.5 *In situ hybridisation*

In situ hybridisation has been used in this thesis to identify and quantify levels of RNA. Radioisotope-labelled antisense oligonucleotides corresponding to a section of the gene sequence of interest are hybridised to tissue sections. Exposure to photographic film results in an image being produced on the film. Additionally, sections can be dipped in photographic emulsion for more detailed examination of cellular localisation and distribution.

A.5.1 *Tissue preparation*

- Remove brains and freeze in isopentane (Sigma) at –45 ± 5 °C.
- Cut sections on cryostat at 15 μm and thaw-mount onto poly-L-lysine-coated slides (BDH).
- Airdry for 1–2 h.
- Fix in freshly made, sterile, ice cold 4 % PFA in 0.1 M PBS for 5 min.
- Rinse in sterile PBS for 1 min.
- Dehydrate in sterile 70 % ethanol for 5 min.
- Store under sterile 95 % ethanol in cold room until hybridisation.

A.5.2 *Probe labelling*

- Dilute oligonucelotides to 5 ng/μl.
- Set up labelling reaction:
  - Oligonucleotide (5 ng/μl) 2.0 μl
  - rTdT 5 x buffer (Promega) 2.5 μl
  - rTdT (5 u/μl; Promega) 1.5 μl

- ^{35}S dATP (Perkin-Elmer) 1.5 μl
- dH_2O 5.0 μl
- **Total** **12.5 μl**

- Incubate 1 h at 37 °C.
- Quench reaction with 40 μl TE[1].
- Purify using column (Bio-Rad).
- Add 2 μl to 4 ml scintillation fluid and check on scintillation counter. Count required >2000000 cpm / 2 μl.
- Add 1 μl DTT to labelled probe.
- Store at –20 °C.

A.5.3 Hybridisation to sections

- Remove slides from alcohol and allow to air dry.
- Prewarm hybridisation buffer[2] to 42 °C.
- Prepare hybridisation chamber with filter paper soaked in 50 % formamide and 5 x SSC buffer.
- Add 1 μl of probe to 100 μl hybridisation buffer per slide.
- Add 2 μl of DTT per 100 μl hybridisation mix and vortex.
- Prepare non-specific binding (NSB) control mix by adding 2 μl of unlabelled probe per 100 μl hybridisation mix.

[1]TE: 10 mM Tris pH 8, 1 mM EDTA, pH 8; Sigma

[2]Hybridisation buffer: 50 % formamide (Sigma), 4 x SSC (National Diagnostics), 10 $ dextran sulphate, 5 x Denhardt's, 200 μg/ml acid-alkali cleaved salmon sperm DNA, 100 μg/ml long chain polyadenylic acid, 120 μg/ml heparin, 25 mM sodium phosphate ph 7.0, 1 mM sodium pyrophosphate

- Apply 100 μl of hybridisation mix to each slide and place a coverslip on top making sure the liquid covers all sections.
- Seal lid of the chamber with parafilm and leave overnight at 42 °C.
- Remove coverslips and wash slides twice in 1 x SSC at 55 °C for 30 min.
- Wash slides in 0.1 x SSC for 1 min at room temperature.
- Dehydrate sections in 70 % ethanol for 1 min RT follwed by 95 % ethanol for 1 min at RT.
- Airdry for 1–2 h.
- Expose to autoradiographic film for 1 week.

A.5.4 Emulsion dipping in situ *hybridised sections*

All procedures must be carried out in dark room under safe lights.

- Warm 15 ml of 0.5 % glycerol to 42 °C in a Falcon tube.
- Add emulsion gel (K5; Ilford, Mobberley, UK) up to a volume of 30 ml, making a 1:1 ratio with glycerol.
- Melt emulsion at 42 °C for 30 min.
- Gently invert tube to ensure thorough mixing of emulsion.
- Individually dip slides into emulsion using dipping chamber and drain excess off. Allow slides to dry in a humid atmosphere for 3–4 h.
- Transfer slides to light-tight boxes containing some silica gel wrapped in tissue paper. Seal the box and store at 4 °C until ready for developing.
- When developing the slides transfer to glass racks under safe light conditions.
- Immerse in 250 ml developer (Kodak D19; Sigma) at 22°C for 2 min.

- Rinse briefly in dH_2O for 30 s.
- Transfer to fix for 4 min.
- Rinse twice in dH_2O for 15 min.

Lights can be turned on at this point

- Counterstain for 2 min in thionin stain[3].
- Dehydrate through ethanol series and Histoclear, mount and coverslip.

[3]Thionin stain: 2.5 ml 1 % thionin, 2.5 ml 1 M sodium acetate and 2.5 ml 1.2 M acetic acid, 242.5 ml dH_2O

A.6 Corticosterone assay

- Add 10 μl of plasma to 500 μl of acidic citrate buffer[1].
- Load in triplicate (100 μl per tube) with 50 μl radioactive tracer (^{125}I-corticosterone, 0.0036 MBq/ml, Oxford Bioinnovation, UK) and 50 μl specific antibody (courtesy of Dr Gabor Makara, Institute of Experimental Medicine, Budapest Hungary) and vortex.
- Prepare sample standard curve with serially diluted corticosterone standards (Sigma). Also include tubes with pure tracer (50 μl), tracer (50 μl) plus buffer (100 μl), and quality control samples (from CORT stock, Sigma).
- Cover and incubate overnight at 4 °C.
- Add 500 μl activated charcoal solution[2] and centrifuge at 4,000 rpm for 15 min at 4 °C.
- Measure radioactivity in each sample using gamma counter.

[1]Citrate buffer: 14.6 g tri-sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), BDH, UK; 13.8 g sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4 \cdot H_2O$), BDH, UK; bovine serum albumin, 2 g, Sigma, UK, pH to 3.0 with HCl, made to 2 l volume with deionised water

[2]Activated charcoal solution: dextran T70 (0.05 mg/100 ml buffer; Amersham Parmacia Biotech AB, Sweden), activated charcoal (5 g/100 ml buffer; Sigma, UK)